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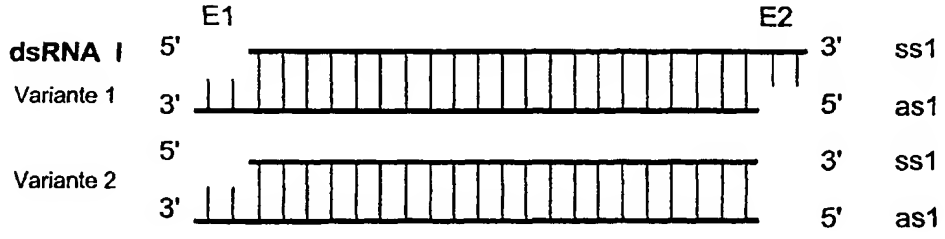
[Fortsetzung auf der nächsten Seite]

(54) Title: METHOD FOR INHIBITING THE EXPRESSION OF A TARGET GENE

(54) Bezeichnung: VERFAHREN ZUR HEMMUNG DER EXPRESSION EINE ZIELGENS



(57) Abstract: The invention relates to a method for inhibiting the expression of a target gene in a cell, comprising the following steps: introduction of an amount of at least one dual-stranded ribonucleic acid (dsRNA I) which is sufficient to inhibit the expression of the target gene. The dsRNA I has a dual-stranded structure formed by a maximum of 49 successive nucleotide pairs. One strand (as1) or at least one section of the one strand (as1) of the dual-stranded structure is complementary to the sense strand of the target gene. The dsRNA has an overhang on the end (E1) of dsRNA I formed by 1 - 4 nucleotides.

(57) Zusammenfassung: Die Erfindung betrifft ein Verfahren zur Hemmung der Expression eines Zielgens in einer Zelle umfassend die folgenden Schritte: Einführen mindestens einer doppelstängigen Ribonukleinsäure (dsRNA I) in einer zur Hemmung der Expression des Zielgens ausreichenden Menge, wobei die dsRNA I eine doppelsträngige aus höchstens 49 aufeinanderfolgenden Nukleotidpaaren gebildete Struktur aufweist, und wobei ein Strang (as1) oder zumindest ein Abschnitt des einen Strangs (as1) der doppelsträngigen Struktur komplementär zum Sinn-Strang des Zielgens ist, und wobei die dsRNA am einen Ende (E1) der dsRNA I einen aus 1 bis 4 Nukeotiden gebildeten überhang aufweist.

Veröffentlicht:

— *ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts*

Zur Erklärung der Zweibuchstaben-Codes und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

Verfahren zur Hemmung der Expression eines Zielgens

Die Erfindung betrifft ein Verfahren, eine Verwendung und ein Medikament zur Hemmung der Expression eines Zielgens.

Aus der WQ 99/32619 sowie der WO 00/44895 sind Verfahren zur Hemmung der Expression von medizinisch oder biotechnologisch interessanten Genen mit Hilfe einer doppelsträngigen Ribonukleinsäure (dsRNA) bekannt. Die bekannten Verfahren sind zwar hoch effektiv. Es besteht gleichwohl das Bedürfnis, deren Effizienz weiter zu steigern.

Aufgabe der vorliegenden Erfindung ist es, die Nachteile nach dem Stand der Technik zu beseitigen. Es sollen insbesondere ein Verfahren, eine Verwendung und ein Medikament angegeben werden, mit denen eine noch effizientere Hemmung der Expression eines Zielgens erreichbar ist.

Diese Aufgabe wird durch die Merkmale der Ansprüche 1, 41 und 81 gelöst. Vorteilhafte Ausgestaltungen ergeben sich aus den Merkmalen der Ansprüche 2 bis 40, 42 bis 80 und 82 bis 120.

Mit den erfindungsgemäß beanspruchten Merkmalen wird überraschenderweise eine drastische Erhöhung der Effektivität der Hemmung der Expression eines Zielgens in vitro und in vivo erreicht. Durch die besondere Ausbildung der Enden der dsRNA kann sowohl deren Effizienz bei der Vermittlung der hemmenden Wirkung auf die Expression eines Zielgens als auch deren Stabilität gezielt beeinflusst werden. Durch die Vergößerung der Stabilität wird die wirksame Konzentration in der Zelle erhöht.

Unter einem "Zielgen" im Sinne der Erfindung wird der DNA-Strang der doppelsträngigen DNA in der Zelle verstanden, welcher koplementär zu einem bei der Transkription als Matritze dienenden DNA-Strang einschließlich aller transkibierten Be-

reiche ist. Bei dem "Zielgen" handelt es sich also im allgemeinen um den Sinnstrang. Der eine Strang bzw. Antisinnstrang (as1) kann komplementär zu einem bei der Expression des Zielgens gebildeten RNA-Transkipt oder deren Prozessierungsprodukt, z.B. eine mRNA, sein. Unter "Einführen" wird die Aufnahme in die Zelle verstanden. Die Aufnahme kann durch die Zelle selbst erfolgen; sie kann auch durch Hilfsstoffe oder Hilfsmittel vermittelt werden. Unter einem "Überhang" wird ein endständiger einzelsträngiger Überstand verstanden, welcher nicht nach Watson & Crick gepaarte Nukleotide aufweist. Unter einer "doppelsträngigen Struktur" wird eine Struktur verstanden, bei der die Nukleotide der Einzelstränge im Wesentlichen nach Watson & Crick gepaart sind. Im Rahmen der vorliegenden Erfindung kann eine doppelsträngige Struktur auch einzelne Fehlpaarungen ("Mismatches") aufweisen.

Nach einer besonderes vorteilhaften Ausgestaltung weist die dsRNA I den Überhang am 3'-Ende des einen Strangs bzw. Antisinnstrangs as1 und/oder am 3'-Ende des anderen Strangs bzw. Sinnstrang ss1 auf. Die dsRNA I kann auch an einem Ende glatt ausgebildet sein. In diesem Fall befindet sich das glatte Ende vorteilhafterweise auf der Seite der dsRNA I, die das 5'-Ende des einen Strangs (Antsinnstrang; as1). In dieser Ausbildung zeigt die dsRNA I einerseits eine sehr gute Effektivität und andererseits eine hohe Stabilität im lebenden Organismus. Die Effektivität insgesamt in vivo ist hervorragend. Der Überhang ist zweckmäßigerweise aus 1 bis 4 Nukleotiden, vorzugsweise aus 1 oder 2 Nukleotiden, gebildet.

Nach einem weiteren Ausgestaltungsmerkmal kann die Effektivität des Verfahrens weiter erhöht werden, wenn zumindest eine entsprechend der erfindungsgemäßen dsRNA I ausgebildete weitere dsRNA II in die Zelle eingeführt wird, wobei der eine Strang oder zumindest ein Abschnitt des einen Strangs der doppelsträngigen Struktur der dsRNA I komplementär zu einem ersten Bereich des Sinnstrangs des Zielgens ist, und wobei

ein weiterer Strang oder zumindest ein Abschnitt des weiteren Strangs der doppelsträngigen Struktur der weiteren dsRNA II komplementär zu einem zweiten Bereich des Sinnstrangs des Zielgens ist. Die Hemmung der Expression des Zielgens ist in diesem Fall deutlich gesteigert. Der erste und der zweite Bereich können abschnittsweise überlappen, aneinander grenzen oder auch voneinander beabstandet sein.

Es hat sich weiter als vorteilhaft erwiesen, wenn die dsRNA I und/oder die weitere dsRNA II eine Länge von weniger als 25 aufeinander folgenden Nukleotidpaaren aufweisen. Als besonders effektiv hat sich eine Länge im Bereich zwischen 19 und 23 Nukleotidpaaren erwiesen. Die Effizienz kann weiter gesteigert werden, wenn an den vorzugsweise aus 19 bis 23 Nukleotidpaaren gebildeten Doppelsträngen einzelsträngige Überhänge von 1 bis 4 Nukleotiden vorhanden sind.

Das Zielgen kann nach einem weiteren Ausgestaltungsmerkmal eine der in dem anhängenden Sequenzprotokoll wiedergegebenen Sequenzen SQ001 bis SQ140 aufweisen. Es kann auch aus der folgenden Gruppe ausgewählt sein: Onkogen, Cytokin-Gen, Id-Protein-Gen, Priongen, Gene zur Expression von Angiogenese induzierenden Molekülen, von Adhäsions-Molekülen und Zelloberflächenrezeptoren, Gene von Proteinen, die an metastasierenden und/oder invasiven Prozessen beteiligt sind, Gene von Proteinasen sowie Apoptose- und Zellzyklus-regulierende Molekülen sowie Gene zur Expression des EGF-Rezeptors. Beim Zielgen kann es sich insbesondere um das MDR1-Gen handeln. Es kann in diesem Zusammenhang eine der Sequenzen SQ141 - 173 bestehende bzw. ein aus jeweils zusammengehörenden Antisinn (as)- und Sinnsequenzen (ss) kombinierte dsRNA I/II verwendet werden.

Nach einem weiteren vorteilhaften Ausgestaltungsmerkmal wird die Expression nach dem Prinzip der RNA-Interferenz gehemmt.

Das Zielgen wird zweckmäßigerweise in pathogenen Organismen, vorzugsweise in Plasmodien, exprimiert. Es kann Bestandteil eines Virus oder Viroids, insbesondere eines humanpathogenen Virus oder Viroids, sein. Das Virus oder Viroid kann auch ein tier- oder pflanzenpathogenes Virus oder Viroid sein.

Nach einem weiteren Ausgestaltungsmerkmal ist vorgesehen, dass die ungepaarten Nukleotide durch Nukleosidthiophosphate substituiert sind.

Zumindest ein Ende der dsRNA I/II kann modifiziert werden, um einem Abbau in der Zelle oder einer Dissoziation in die Einzelstränge entgegenzuwirken. Vorteilhafterweise wird dazu der durch die komplementären Nukleotidpaare bewirkte Zusammenhalt der doppelsträngigen Struktur durch mindestens eine chemische Verknüpfung erhöht. Die chemische Verknüpfung kann durch eine kovalente oder ionische Bindung, eine Wasserstoffbrückenbindung, hydrophobe Wechselwirkungen, vorzugsweise van-der-Waals- oder Stapelungswechelwirkungen, oder durch Metall-Ionenkoordination gebildet werden. Es hat sich weiter als zweckmäßig und die Stabilität erhöhend erwiesen, wenn die chemische Verknüpfung in der Nähe des einen Endes gebildet ist. Weitere vorteilhafte Ausgestaltungen hinsichtlich der chemischen Verknüpfung können den Merkmalen der Ansprüche 24 bis 30 entnommen werden, ohne dass es dafür einer näheren Erläuterung bedarf.

Die dsRNA I/II kann dann besonders einfach in die Zelle eingeschleust werden, wenn sie in micellare Strukturen, vorteilhafterweise in Liposomen, eingeschlossen wird. Zum Transport der dsRNA I/II in die Zelle hat es sich auch als vorteilhaft erwiesen, dass diese an mindestens ein von einem Virus stammendes, davon abgeleitetes oder ein synthetisch hergestelltes virales Hüllprotein gebunden, damit assoziiert oder davon umgeben werden. Das Hüllprotein kann vom Polyomavirus abgeleitet sein. Das Hüllprotein kann insbesondere das Virus-Protein

1 und/oder das Virus-Protein 2 des Polyomavirus enthalten. Nach einer weiteren Ausgestaltung ist vorgesehen, dass bei Bildung eines Kapsids oder kapsidartigen Gebildes aus dem Hüllprotein die eine Seite zum Inneren des Kapsids oder kapsidartigen Gebildes gewandt ist. Ferner ist es von Vorteil, dass der eine Strang der dsRNA I/II (as1/2) zum primären oder prozessierten RNA-Transkript des Zielgens komplementär ist. Die Zelle kann eine Vertebratenzelle oder eine menschliche Zelle sein.

Weiterhin hat es sich gezeigt, dass die dsRNA I/II vorteilhafterweise bereits in einer Menge von höchstens 5 mg/kg Körpergewicht pro Tag einem Säugetier, vorzugsweise einem Menschen, verabreicht werden kann. Bereits in dieser geringen Dosis wird eine ausgezeichnete Effektivität erzielt.

Überraschenderweise hat sich gezeigt, dass die dsRNA I/II zur Applikation in eine Pufferlösung aufgenommen und dann oral oder mittels Injektion oder Infusion intravenös, intratumoral, inhalativ, intraperitoneal verabreicht werden kann.

Erfindungsgemäß ist weiterhin die Verwendung einer doppelsträngigen Ribonukleinsäure (dsRNA I) zur Hemmung der Expression eines Zielgens in einer Zelle vorgesehen, wobei die dsRNA I eine doppelsträngige aus höchstens 49 aufeinander folgenden Nukleotidpaaren gebildete Struktur aufweist, und wobei ein Strang (Antisinnstrang; as1) oder zumindest ein Abschnitt des einen Strangs (as1) der doppelsträngigen Struktur komplementär zum Sinnstrang des Zielgens ist, und wobei die dsRNA I zumindest an einem Ende einen aus 1 bis 4 Nukleotiden gebildeten Überhang aufweist.

Nach weiterer Maßgabe der Erfindung ist ein Medikament zur Hemmung der Expression eines Zielgens in einer Zelle vorgesehen, enthaltend eine doppelsträngige Ribonukleinsäure (dsRNA I) in einer zur Hemmung der Expression des Zielgens ausrei-

tung unter dem Fluoreszenzmikroskop erfolgte frühestens 3 Stunden nach Injektion anhand der grünen Fluoreszenz.

Vorbereitung der Zellkulturen:

Die Kultivierung der Zellen erfolgte in DMEM mit 4,5 g/l Glucose, 10 % fötalem Kälberserum (FCS), 2 mM L-Glutamin, Penicillin/Streptomycin (100 IE/100 µg/ml, Biochrom) im Brutschrank unter 5 % CO_2-Atmosphäre bei 37°C. Die Zellen wurden alle 3 Tage passagiert, um sie in der exponentiellen Wachstumsphase zu halten. Einen Tag vor der Durchführung der Transfektion wurden die Zellen trypsiniert (10x Trypsin/TEDTA, Biochrom) und mit einer Zelldichte von 0,3 x 10^5 Zellen in beschichteten Petrischalen (CORNING® Cell Culture Dish, 35 mm, Corning Inc., Corning, USA) ausgesät. Die Petrischalen wurden mit 0,2 % Gelatine (Biochrom) für mindestens 30 Minuten bei 37°C inkubiert, einmal mit PBS gewaschen und sofort für die Aussaat der Zellen verwendet. Um ein Wiederfinden individueller Zellen zu ermöglichen, wurden CELLocate Coverslips der Fa. Eppendorf (Square size 55 µm) verwendet.

Mikroinjektion:

Zur Durchführung der Mikroinjektion wurden die Petrischalen ca. 10 Minuten aus dem Brutschrank genommen. Pro Schale und Ansatz wurden ca. 50 Zellen mikroinjiziert (FemtoJet; Mikromanipulator 5171, Eppendorf). Für die Mikroinjektion wurden Glaskapillaren (FemtoTip) der Firma Eppendorf mit einem Spitzeninnendurchmesser von 0,5 µm verwendet. Die Injektionsdauer betrug 0,8 Sekunden und der Druck 30 hPa. Durchgeführt wurden die Mikroinjektionen an einem Olympus IX50 Mikroskop mit Fluoreszenzeinrichtung. Als Injektionspuffer wurde 14 mM NaCl, 3 mM KCl, 10 mM KH_2PO_4, pH 7,0 verwendet, der 0,01 µg/µl pcDNA-YFP enthielt. Zur Überprüfung einer erfolgreichen Mikroinjektion wurde der Injektionslösung jeweils 0,08% (w/v) an Dextran-70000 gekoppeltes Texas-Rot (Molecular Probes, Leiden, Niederlande) zugesetzt. Um die Inhibition der YFP-Expression mit spezifischer dsRNA zu untersuchen, wurden der

Injektionslösung dsRNAs zugegeben: Ansatz 1: 0,1 µM dsRNA (Sequenzprotokoll SQ148/149); Ansatz 2: 0,1 µM dsRNA (Sequenzprotokoll SQ148/159); Ansatz 3: ohne RNA. Nach der Mikroinjektion wurden die Zellen für mindestens drei weitere Stunden im Brutschrank inkubiert. Danach wurden die intrazelluläre YFP-Fluoreszenz am Mikroskop ausgewertet:
gleichzeitig rot und grün-fluoreszierende Zellen: Mikroinjektion war erfolgreich, es wird keine Inhibition der YFP-Expression durch dsRNA beobachtet; bzw. es handelt sich um Kontrollzellen, in die keine dsRNA injiziert wurde;
nur rot-fluoreszierende Zellen: Mikroinjektion war erfolgreich, die dsRNA inhibiert YFP-Expression.

Ergebnisse:
Bei einer dsRNA-Konzentration von 0,1 µM konnte beim Einsatz der dsRNA mit den an beiden 3´-Enden um je zwei Nukleotide überstehenden Einzelstrangbereichen (Sequenzprotokoll SQ148/159) eine merklich erhöhte Hemmung der Expression des YFP-Gens in Fibroblasten beobachtet werden im Vergleich zur dsRNA ohne überstehende Einzelstrangenden (Tabelle 1).

Die Verwendung von kurzen, 19-25 Basenpaare enthaltenden, dsRNA-Molekülen mit Überhängen aus wenigen, vorzugsweise 1 bis 3 nicht-basengepaarten, einzelsträngigen Nukleotiden ermöglicht somit eine vergleichsweise stärkere Hemmung der Genexpression in Säugerzellen als die Verwendung von dsRNAs mit derselben Anzahl von Basenpaaren ohne die entsprechenden Einzelstrangüberhänge bei jeweils gleichen RNA-Konzentrationen.

Ansatz	Name	Sequenzprotokoll-Nr.	0.1 µM
1	S1A/ S1B	SQ148 SQ149	+
2	S1A/ S4B	SQ148 (überstehende Enden) SQ159	+++
3		ohne RNA	-

Tabelle 1: Die Symbole geben den relativen Anteil an nicht oder schwach grün-fluoreszierenden Zellen an (+++ > 90%; ++ 60-90%; + 30-60%; - < 10%).

II. Hemmung der Genexpression eines Zielgens in kultivierten HELA-S3-Zellen und Mausfibroblasten durch dsRNA:

Die Effektivität der Inhibition der YFP-Expression nach transienter Transfektion eines YFP-codierenden Plasmids auf der Basis der RNA-Interferenz mit dsRNAs läßt sich durch Gestaltung der 3´-Enden und der Länge des basengepaarten Bereichs modulieren.

Ausführungsbeispiel:

Zum Wirksamkeitsnachweis der dsRNA bei der spezifischen Inhibition der Genexpression wurden transient transfizierte NIH/3T3-Zellen (Fibroblasten aus NIH Swiss Mausembryo, ECCAC (European collection of animal cell culture) Nr. 93061524) und HELA-S3 (humane cervikale Karzinomzellen, DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) Nr. ACC 161) verwendet. Für die Transfektion wurde das Plasmid pcDNA-YFP verwendet, das ein 800 bp großes Bam HI /Eco RI-YFP-Fragment in den entsprechenden Schnittstellen des Vektors pcDNA3 enthält. Aus der Sequenz des gelb-fluoreszierenden Proteins (YFP) abgeleitete doppelsträngige RNAs (dsRNAs) wurden herge-

stellt und zusammen mit dem Plasmid pcDNA-YFP transient in die Fibroblasten transfiziert (Die verwendeten spezifischen dsRNAs sind in ihren Antisinn-Strängen komplementär zu entsprechenden Abschnitten der Gensequenzen von sowohl YFP als auch GFP). Nach 48 Stunden wurde die Fluoreszenzabnahme quantifiziert. Als Kontrollen fungierten Zellen, die entweder nur mit pcDNA-YFP oder mit pcDNA-YFP und einer Kontroll-dsRNA (nicht aus der YFP-Sequenz abgeleitet) transfiziert wurden.

Versuchsprotokoll:

dsRNA-Synthese:

Mittels eines RNA-Synthesizers (Typ Expedite 8909, Applied Biosystems, Weiterstadt, Deutschland) und herkömmlicher chemischer Verfahren wurden die aus den Sequenzprotokollen ersichtlichen RNA-Einzelstränge und die zu ihnen komplementären Einzelstränge synthetisiert. Anschließend erfolgte die Reinigung der rohen Syntheseprodukte mit Hilfe der HPLC. Verwendet wurde die Säule NucleoPac PA-100, 9x250 mm, der Fa. Dionex; als Niedersalz-Puffer 20 mM Tris, 10 mM $NaClO_4$, pH 6,8, 10% Acetonitril und als Hochsalz-Puffer 20 mM Tris, 400 mM $NaClO_4$, pH 6,8, 10% Acetonitril. Der Fluß betrug 3 ml/ Minute. Die Hybridisierung der Einzelstränge zum Doppelstrang erfolgte durch Erhitzen des stöchiometrischen Gemischs der Einzelstränge in 10 mM Natriumphosphatpuffer, pH 6,8, 100 mM NaCl, auf 80-90°C und nachfolgendes langsames Abkühlen über 6 Stunden auf Raumtemperatur.

Aussaat der Zellen:

Alle Zellkulturarbeiten wurden unter sterilen Bedingungen in einer entsprechenden Werkbank (HS18, Hera Safe, Kendro, Heraeus) durchgeführt. Die Kultivierung der NIH/3T3-Zellen und der HELA-S3 erfolgte im Brutschrank (CO_2-Inkubator T20, Hera cell, Kendro, Heraeus) bei 37°C, 5% CO_2 und gesättigter

Luftfeuchtigkeit in DMEM (Dulbecco`s modified eagle medium, Biochrom), für die Mausfibroblasten, und Ham`s F12 für die HELA-Zellen mit 10% FCS (fetal calf serum, Biochrom), 2 mM L-Glutamin (Biochrom) und Penicillin/Streptomycin (100 IE/100 µg/ml, Biochrom). Um die Zellen in der exponentiellen Wachstumsphase zu halten, wurden die Zellen alle 3 Tage passagiert. 24 Stunden vor der Durchführung der Transfektion wurden die Zellen trypsiniert (10x Trypsin/EDTA, Biochrom, Deutschland) und mit einer Zelldichte von 1,0 x 10^4 Zellen/Vertiefung in einer 96-Loch-Platte (Multiwell Schalen 96-Well Flachboden, Labor Schubert & Weiss GmbH) in 150 µl Wachstumsmedium ausgesät.

Durchführung der transienten Transfektion:

Die Transfektion wurde mit Lipofectamine Plus™ Reagent (Life Technologies) gemäß den Angaben des Herstellers durchgeführt. Pro Well wurden 0,15 µg pcDNA-YFP-Plasmid eingesetzt. Das Gesamt-Transfektionsvolumen betrug 60 µl. Es wurden jeweils3-fach-Proben angesetzt. Die Plasmid-DNA wurde zuerst zusammen mit der dsRNA komplexiert. Dazu wurde die Plasmid-DNA und die dsRNA in serumfreiem Medium verdünnt und pro 0,1 µg Plasmid-DNA 1 µl PLUS Reagent eingesetzt (in einem Volumen von 10 µl) und nach dem Mischen für 15 Minuten bei Raumtemperatur inkubiert. Während der Inkubation wurde pro 0,1 µg Plasmid-DNA 0,5 µl Lipofectamine in insgesamt 10 µl serumfreiem Medium verdünnt, gut gemischt, zu dem Plasmid/dsRNA/PLUS-Gemisch zugegeben und nochmals 15 Minuten inkubiert. Während der Inkubation wurde ein Mediumwechsel durchgeführt. Die Zellen wurden dazu 1 x mit 200 µl serumfreiem Medium gewaschen und danach mit 40 µl serumfreiem Medium bis zur Zugabe von DNA/dsRNA/PLUS/Lipofectamine weiter im Brutschrank inkubiert. Nach der Zugabe von 20 µl DNA/dsRNA/PLUS/Lipofectamine pro

Well wurden die Zellen für 2,5 Stunden im Brutschrank inkubiert. Anschließend wurden die Zellen nach der Inkubation 1 x mit 200 µl Wachstumsmedium gewaschen und für 24 Stunden bis zur Detektion der Fluoreszenz in 200 µl Wachstumsmedium im Brutschrank inkubiert.

Detektion der Fluoreszenz:

24 Stunden nach dem letzten Mediumwechsel wurde die Fluoreszenz der Zellen am Fluoreszenz-Mikroskop (IX50-S8F2, Fluoreszenz-Einheit U-ULS100Hg, Brenner U-RFL-T200, Olympus) mit einer USH-I02D-Quecksilber-Lampe (USHIO Inc., Tokyo, Japan), ausgestattet mit einem WIB-Fluoreszenz-Würfel und einer digitalen CCD-Kamera (Orca IIIm, Hamamatsu) und C4742-95 Kamera-Controller) photographiert. Die Auswertung der Fluoreszenzaufnahmen erfolgte mit der analysis-Software 3.1 (Soft Imaging Sytem GmbH, Deutschland). Um die YFP-Fluoreszenz in Relation zur Zelldichte zu setzen, wurde eine Zellkernfärbung (Hoechst-Staining) durchgeführt. Dazu wurden die Zellen in 100 µl Methylcarnoy (75% Methanol, 25% Eisessig) zuerst für 5 und danach nochmals für 10 Minuten in Methylcarnoy fixiert. Nach dem Lufttrocknen wurden die fixierten Zellen für 30 Minuten im Dunkeln mit 100 µl pro Well Hoechst-Farbstoff (75 ng/ml) inkubiert. Nach 2maligem Waschen mit PBS (PBS Dulbecco w/o Ca^{2+}, Mg^{2+}, Biochrom) wurden die Hoechst-gefärbten Zellen unter dem Fluoreszenz-Mikroskop (Olympus, WU-Fluoreszenz-Würfel für Hoechst) photographiert.

In den Fig. 3 bis 9 sind die Ergebnisse zur Inhibition der YFP-Expression durch dsRNA in kultivierten Zellen zusammengefasst:

In Fig. 3, 4, 5 und 6 sind die Effekte von YFP-spezifischen dsRNAs und von Kontroll-dsRNAs auf die YFP-Expression in NIH/3T3-Mausfibroblasten nach transienter Transfektion zusammengefasst. Die Experimente wurden wie im Versuchsprotokoll

Die Fig. 10 bis 17 zeigen die Serumstabilität der dsRNA nach Inkubation mit humanem bzw. murinem Serum und nachfolgender elektrophoretischer Auftrennung im 20%igem 7M Harnstoffgel.

Fig. 10: Inkubation von S1 (0-22-0) in Maus-Serum

1. zum Zeitpunkt 0 (ohne Serum)
2. zum Zeitpunkt 0
3. für 30 Minuten
4. für 1 Stunde
5. für 2 Stunden
6. für 4 Stunden
7. für 12 Stunden
8. 2 µl 100 µM S1 ohne Inkubation

S1A) Sinnstrang S1 (10 µl 20 µM S1A)

S1B) Antisinnstrang S1 (10 µl 20 µM S1B)

Fig. 11: Inkubation von S1 (0-22-0) in humanem Serum

1. 2 µl 100 µM S1 unbehandelt (ohne Inkubation)
2. für 30 Minuten
3. für 2 Stunden
4. für 4 Stunden
5. für 6 Stunden
6. für 8 Stunden
7. für 12 Stunden
8. für 24 Stunden

S1A) Sinnstrang S1 (10 µl 20 µM S1A)

S1B) Antisinnstrang S1 (10 µl 20 µM S1B)

Fig. 12: Inkubation von S7 (2-19-2) in Maus-Serum

1. zum Zeitpunkt 0 (ohne Serum)
2. für 30 Minuten
3. für 4 Stunden
4. für 12 Stunden

Fig. 13: Inkubation von S7 (2-19-2) in humanem Serum

1. Sinnstrang S7 (10 µl 20 µM S7A)

2. Antisinnstrang S7 (10 µl 20 µM S7B)
3. für 30 Minuten
4. für 1 Stunde
5. für 2 Stunden
6. für 4 Stunden
7. für 6 Stunden
8. für 12 Stunden
9. für 24 Stunden
10. zum Zeitpunkt 0 (ohne Serum)

Fig. 14: Inkubation von K3 (2-19-2) in Maus-Serum

1. Sinnstrang K3 (10 µl 20 µM K3A)
2. Antisinnstrang K3 (10 µl 20 µM K3B)
3. zum Zeitpunkt 0 (ohne Serum)
4. zum Zeitpunkt 0 (mit Serum)
5. für 30 Minuten
6. für 1 Stunde
7. für 2 Stunden
8. für 4 Stunden
9. für 12 Stunden

Fig. 15: Inkubation von PKC1/2 (0-22-2) in Maus-Serum

1. für 30 Minuten
2. für 1 Stunde
3. für 2 Stunden
4. für 4 Stunden
5. für 12 Stunden
6. 2 µl 100 µM PKC1/2 (unbehandelt)

Fig. 16: Inkubation von S1A/S4B (0-22-2) in humanem Serum

1. zum Zeitpunkt 0 (ohne Serum)
2. für 24 Stunden
3. für 12 Stunden
4. für 8 Stunden
5. für 6 Stunden
6. für 4 Stunden

7. für 2 Stunden
8. für 30 Minuten
9. Sinnstrang S1A (10 µl 20 µM S1A)
10. Antisinnstrang S4B (10 µl 20 µM S4B)

Fig. 17: Inkubation von K2 (2-22-2) in humanem Serum

1. Sinnstrang K2 (10 µl 20 µM K2A)
2. Antisinnstrang K2 (10 µl 20 µM K2B)
3. zum Zeitpunkt 0 (ohne Serum)
4. für 30 Minuten
5. für 2 Stunden
6. für 4 Stunden
7. für 6 Stunden
8. für 8 Stunden
9. für 12 Stunden
10. für 24 Stunden

Ergebnisse:

dsRNAs ohne einzelsträngige Bereiche an den 3´-Enden sind im Serum sowohl von Mensch und Maus wesentlich stabiler als dsRNAs mit einzelsträngigen 2nt-Überhängen an den 3´-Enden (Fig. 10 bis 14 und 17). Nach 12 bzw. 24 Stunden Inkubation von S1 in murinem bzw. humanem Serum ist noch immer eine Bande in der ursprünglichen Größe fast vollständig erhalten. Dagegen nimmt bei dsRNAs mit 2nt-Überhängen an beiden 3´-Enden die Stabilität in humanem als auch im murinen Serum deutlich ab. Bereits nach 4 Stunden Inkubation von S7 (Fig. 12 und 13) oder K3 (Fig. 14) ist keine Bande in der Originalgröße mehr detektierbar.

Um die Stabilität von dsRNA im Serum zu erhöhen, ist es ausreichend, wenn die dsRNA ein glattes Ende besitzt. Im Maus-Serum ist nach 4 Stunden Inkubation (Fig. 15, Bahn 4) die

Bande in der Originalgröße kaum abgebaut im Vergleich zu S7 (nach 4 Stunden vollständiger Abbau; Fig. 12, Bahn 3).

Als optimaler Kompromiß hinsichtlich der biologischen Wirksamkeit von dsRNA kann die Verwendung von dsRNA mit einem glattem Ende und einem einzelsträngigem Bereich von 2 Nukleotiden angesehen werden, wobei sich der einzelsträngige Überhang am 3´-Ende des Antisinn-Stranges befinden sollte.

Die hier verwendeten Sequenzen sind aus der nachstehenden Tabelle 2 und den Sequenzprotokollen SQ148-151 und 153-167 ersichtlich.

Name	Sequenz-protokoll-Nr.	dsRNA-Sequenz	
S1	SQ148 SQ149	(A) 5´- CCACAUGAAGCAGCACGACUUC -3´ (B) 3´- GGUGUACUUCGUCGUGCUGAAG -5´	0-22-0
S7	SQ150 SQ151	(A) 5´- CCACAUGAAGCAGCACGACUU -3´ (B) 3´- CUGGUGUACUUCGUCGUGCUG -5´	2-19-2
K1	SQ153 SQ154	(A) 5´- ACAGGAUGAGGAUCGUUUCGCA -3´ (B) 3´- UGUCCUACUCCUAGCAAAGCGU -5´	0-22-0
K3	SQ155 SQ156	(A) 5´-GAUGAGGAUCGUUUCGCAUGA-3´ (B) 3´-UCCUACUCCUAGCAAAGCGUA-5´	2-19-2
K2	SQ157 SQ158	(A) 5´- ACAGGAUGAGGAUCGUUUCGCAUG -3´ (B) 3´- UCUGUCCUACUCCUAGCAAAGCGU -5´	2-22-2
S1A/ S4B	SQ148 SQ159	(A) 5´- CCACAUGAAGCAGCACGACUUC -3´ (B) 3´- CUGGUGUACUUCGUCGUGCUGAAG -5´	0-22-2

PKC 1/2	SQ160 SQ161	(A) 5´- CUUCUCCGCCUCACACCGCUGCAA -3´ (B) 3´- GAAGAGGCGGAGUGUGGCGACG -5´	2-22-0
S7/S12	SQ150 SQ162	(A) 5´- CCACAUGAAGCAGCACGACUU -3´ (B) 3´- GGUGUACUUCGUCGUGCUGAA -5´	0-21-0
S7/S11	SQ150 SQ163	(A) 5´- CCACAUGAAGCAGCACGACUU -3´ (B) 3´- CUGGUGUACUUCGUCGUGCUGAA -5´	0-21-2
S13	SQ164 SQ165	(A) 5´- CCACAUGAAGCAGCACGACU -3´ (B) 3´- CUGGUGUACUUCGUCGUGCUGA -5´	0-20-2
S13/14	SQ164 SQ166	(A) 5´- CCACAUGAAGCAGCACGACU -3´ (B) 3´- GGUGUACUUCGUCGUGCUGA -5´	0-20-0
S4	SQ167 SQ159	(A) 5´- CCACAUGAAGCAGCACGACUUCUU -3´ (B) 3´- CUGGUGUACUUCGUCGUGCUGAAG -5´	2-22-2
K1A/ K2B	SQ153 SQ158	(A) 5´- ACAGGAUGAGGAUCGUUUCGCA -3´ (B) 3´- UCUGUCCUACUCCUAGCAAAGCGU -5´	0-22-2
K1B/ K2A	SQ154 SQ157	(A) 5´- ACAGGAUGAGGAUCGUUUCGCAUG -3´ (B) 3´- UGUCCUACUCCUAGCAAAGCGU -5´	2-22-0
S1B/ S4A	SQ149 SQ167	(A) 5´- CCACAUGAAGCAGCACGACUUCUU -3´ (B) 3´- GGUGUACUUCGUCGUGCUGAAG -5´	2-22-0

Tabelle 2

IV. In vivo-Studie:

Es wurde „GFP-Labormäusen", die das Grün-fluoreszierende Protein (GFP) in allen Proteinbiosynthese betreibenden Zellen exprimieren, doppelsträngige RNA (dsRNA), die aus der GFP-Sequenz abgeleitet wurde, bzw. unspezifische dsRNA intravenös in die Schwanzvene injiziert. Am Versuchsende wurden die Tie-

ta Cruz Biotechnology) in einer Verdünnung von 1:1000 erfolgte für 1h bei RT. Danach wurde 3 x 5 min gewaschen und für 1h bei RT mit dem Sekundärantikörper (donkey anti-goat IgG Hoseradish Peroxidase gelabelt, Santa Cruz Biotechnology) in einer Verdünnung von 1 : 10.000 inkubiert. Die Detektion erfolgte mit dem ECL-System von Amersham nach den Angaben des Herstellers.

In den Fig. 18 bis 20 ist die Inhibition der GFP-Expression nach intravenöser Injektion von spezifisch gegen GFP gerichteter dsRNA mit Immunperoxidase-Färbungen gegen GFP an 3 µm Paraffinschnitten dargestellt. Im Versuchsverlauf wurde gegen GFP gerichtete dsRNA mit einem doppelsträngigen Bereich von 22 Nukleotid-(nt)paaren ohne Überhänge an den 3´-Enden (D) und die entsprechende unspezifische Kontroll-dsRNA (B) sowie spezifisch gegen GFP gerichtete dsRNA mit einem 19 Nukleotidpaare umfassenden Doppelstrangbereich mit 2nt-Überhängen an den 3´-Enden (E) und die entsprechende unspezifische Kontroll-dsRNA (C) im 12 Stunden-Turnus über 5 Tage hinweg appliziert. (F) erhielt 1/50 der Dosis von Gruppe D. Als weitere Kontrolle wurden Tiere ohne dsRNA-Gabe (A) bzw. WT-Tiere untersucht. Die Fig. 18 zeigt die Inhibition der GFP-Expression in Nierenschnitten, Fig. 19 in Herz- und Fig. 20 in Pankreasgewebe. In den Fig. 21 bis 23 sind Western Blot-Analysen der GFP-Expression in Plasma und Geweben dargestellt. In der Fig. 21 ist die Inhibition der GFP-Expression im Plasma, in Fig. 22 in der Niere und in Fig. 23 in Herz gezeigt. In Fig. 23 sind Gesamtproteinisolate aus verschiedenen Tieren aufgetragen. Es wurden jeweils gleiche Gesamtproteinmengen pro Bahn aufgetragen. In den Tieren, denen unspezifische Kontroll-dsRNA verabreicht wurde (Tiere der Gruppen B und C), ist die GFP-Expression gegenüber Tieren, die keinerlei dsRNA erhielten, nicht reduziert. Tiere, die spezifisch gegen GFP gerichtete dsRNA mit 2nt-Überhängen an den 3´-Enden

beider Stränge und einen 19 Nukleotidpaare umfassenden Doppelstrangbereich erhielten, zeigten eine signifikant inhibierte GFP-Expression in den untersuchten Geweben (Herz, Niere, Pankreas und Blut), verglichen mit unbehandelten Tieren (Fig. 18 bis 23). Bei den Tieren der Gruppen D und F, denen spezifisch gegen GFP gerichtete dsRNA mit glatten Enden und einem 22 Nukleotidpaare umfassenden Doppelstrangbereich appliziert wurde, zeigten nur jene Tiere, die die dsRNA in einer Dosis von 50 µg/kg Körpergewicht pro Tag erhielten, eine spezifische Inhibition der GFP-Expression, die allerdings weniger deutlich ausgeprägt war als die der Tiere in Gruppe E.

Die zusammenfassende Auswertung von GFP-Inhibition in den Gewebeschnitten und im Western Blot ergibt, dass die Inhibition der GFP-Expression im Blut und in der Niere am stärksten ist (Fig. 18, 21 und 22).

V. Hemmung der Genexpression des EGF-Rezeptors mit dsRNA als therapeutischer Ansatz bei Krebsformen mit EGFR-Überexpression oder EGFR-induzierter Proliferation:

Der Epidermal Growth Factor (=EGF)-Rezeptor (=EGFR) gehört zu den Rezeptor-Tyrosinkinasen, transmembranen Proteinen mit einer intrinsischen Tyrosinkinase-Aktivität, die an der Kontrolle einer Reihe von zellulären Prozessen wie Zellwachstum, Zelldifferenzierungen, migratorischen Prozessen oder der Zellvitalität beteiligt sind (Übersicht in: Van der Geer et al. 1994). Die Familie der EGFR besteht aus 4 Mitgliedern, EGFR (ErbB1), HER2 (ErbB2), HER3 (ErbB3) und HER4 (ErbB4) mit einer transmembranen Domäne, einer cysteinreichen extrazellulären Domäne und einer intrazellullären katalytischen Domäne. Die Sequenz des EGFR, einem 170 kDa Protein, ist seit 1984 bekannt (Ullrich et al., 1984).

Aktiviert wird der EGFR durch Peptid-Wachstumsfaktoren wie EGF, TGFα (transforming growth factor), Amphiregulin, Betacellulin, HB-EGF (heparin-binding EGF-like growth factor) und Neureguline. Ligandenbindung induziert die Bildung von Homo- oder Heterodimeren mit nachfolgender Autophosphorylierung zytoplasmatischer Tyrosine (Ullrich & Schlessinger, 1990; Alroy & Yarden, 1997). Die phosphorylierten Aminosäuren bilden die Bindungsstellen für eine Vielzahl von Proteinen, die an den proximalen Schritten der Signalweiterleitung in einem komplexen Netzwerk beteiligt sind. Der EGFR ist an den verschiedensten Tumorerkrankungen beteiligt und damit ein geeignetes Target für therapeutische Ansätze (Huang & Harari, 1999). Die Mechanismen, die zu einer aberranten EGFR-Aktivierung führen, können auf Überexpression, Amplifikation, konstitutiver Aktivierung mutanter Rezeptor-Formen oder autokrinen Loops beruhen (Voldborg et al., 1997). Eine Überexpression des EGFR wurde für eine Reihe von Tumoren beschrieben, wie z.B. Brustkrebs (Walker & Dearing, 1999), Nicht-Klein-Lungenkarzinom (Fontanini et al., 1998), Pankreaskarzinomen, Kolonkarzinom (Salomon et al., 1995) und Glioblastomen (Rieske et al., 1998). Insbesondere für maligne Glioblastome sind bisher keine effizienten und spezifischen Therapeutika verfügbar.

Ausführungsbeispiel:

Zum Nachweis der Wirksamkeit der dsRNA bei der spezifischen Inhibition der EGFR-Genexpression wurden U-87 MG-Zellen (humane Glioblastomzellen), ECCAC (European collection of animal cell culture) Nr. 89081402, verwendet, die mit spezifisch gegen den EGF-Rezeptor (Sequenzprotokoll SQ 51) gerichteten dsRNA transfiziert wurden. Nach ca. 72 Stunden Inkubation wurden die Zellen geerntet, Protein isoliert und im Western Blot Verfahren die EGFR-Expression untersucht.

Versuchsprotokoll:

dsRNA-Synthese:

Mittels eines RNA-Synthesizers (Typ Expedite 8909, Applied Biosystems, Weiterstadt, Deutschland) und herkömmlicher chemischer Verfahren wurden die aus den Sequenzprotokollen ersichtlichen RNA-Einzelstränge und die zu ihnen komplementären Einzelstränge synthetisiert. Anschließend erfolgte die Reinigung der rohen Syntheseprodukte mit Hilfe der HPLC. Verwendet wurde die Säule NucleoPac PA-100, 9x250 mm, der Fa. Dionex; als Niedersalz-Puffer 20 mM Tris, 10 mM $NaClO_4$, pH 6,8, 10% Acetonitril und als Hochsalz-Puffer 20 mM Tris, 400 mM $NaClO_4$, pH 6,8, 10% Acetonitril. Der Fluß betrug 3 ml/Minute. Die Hybridisierung der Einzelstränge zum Doppelstrang erfolgte durch Erhitzen des stöchiometrischen Gemischs der Einzelstränge in 10 mM Natriumphosphatpuffer, pH 6,8, 100 mM NaCl, auf 80-90°C und nachfolgendes langsames Abkühlen über 6 Stunden auf Raumtemperatur.

Aussaat der Zellen:

Alle Zellkulturarbeiten wurden unter sterilen Bedingungen in einer entsprechenden Werkbank (HS18, Hera Safe, Kendro, Heraeus) durchgeführt. Die Kultivierung der U-87 MG-Zellen erfolgte im Brutschrank (CO_2-Inkubator T20, Hera cell, Kendro, Heraeus) bei 37°C, 5% CO_2 und gesättigter Luftfeuchtigkeit in DMEM (Dulbecco`s modified eagle medium, Biochrom) mit 10% FCS (fetal calf serum, Biochrom), 2 mM L-Glutamin (Biochrom), 1 mM Natrium-Pyruvat (Biochrom), 1xNEAA (Non-essetial Aminoacids, Biochrom) und Penicillin/Streptomycin (100 IE/100 µg/ml, Biochrom). Um die Zellen in der exponentiellen Wachstumsphase zu halten, wurden die Zellen alle 3 Tage passagiert. 24 Stunden vor der Applikation der dsRNA mittels Transfektion wurden die Zellen trypsiniert (10x Trypsin/EDTA,

Biochrom, Deutschland) und mit einer Zelldichte von 5 x 10^5 Zellen/Vertiefung in einer 6-Well-Platte (6-Well Schalen, Labor Schubert & Weiss GmbH) in 1,5 ml Wachstumsmedium ausgesät.

Applikation der dsRNA in kultivierte U-87 MG-Zellen:

Die Applikation der dsRNA erfolgte mittels Transfektion mit dem OLIGOFECTAMINE™ Reagent (Life Technologies) gemäß den Angaben des Herstellers. Das Gesamt-Transfektionsvolumen betrug 1 ml. Zuerst wurde die dsRNA in serumfreiem Medium verdünnt: Dazu wurden pro Well 0,5 µl einer 20 µM Stammlösung spezifisch gegen EGFR gerichteten dsRNA und 9,5 µl einer 20 µM Stammlösung unspezifischer dsRNA (K1A/K2B) mit 175 µl serumfreiem Medium verdünnt (200 nM dsRNA im Transfektionsansatz bzw. 10 nM spezifische EGFR-dsRNA). Das OLIGOFECTAMINE™ Reagent wurde ebenfalls in serumfreien Medium verdünnt: pro Well 3 µl mit 12 µl Medium und danach 10 min bei Raumtemperatur inkubiert. Danach wurde das verdünnte OLIGOFECTAMINE™ Reagent zu den in Medium verdünnten dsRNAs gegeben, gemischt und für weitere 20 min bei RT inkubiert. Während der Inkubation wurde ein Mediumwechsel durchgeführt. Die Zellen wurden dazu 1 x mit 1 ml serumfreiem Medium gewaschen und mit 800 µl serumfreiem Medium bis zur Zugabe von dsRNA/OLIGOFECTAMINE™ Reagent weiter im Brutschrank inkubiert. Nach der Zugabe von 200 µl dsRNA/OLIGOFECTAMINE™ Reagent pro Well wurden die Zellen bis zur Proteinisolierung weiter im Brutschrank inkubiert.

Proteinisolierung:

Ca. 72 Stunden nach der Transfektion wurden die Zellen geerntet und eine Proteinisolierung durchgeführt. Dazu wurde das Medium abgenommen und das Zellmonolayer 1 x mit PBS gewaschen. Nach Zugabe von 200 µl Proteinisolierungspuffer (1x Protease-Inhibitor „Complete", Roche, 50 mM HEPES, pH 7,5,

Kontrolle wurde eine unspezifische dsRNA-Sequenz, die keinerlei Homologie mit der MDR1-Gensequenz aufweist,eingesetzt (K) und eine MOCK-Transfektion durchgeführt, die alle Reagenzien außer dsRNA enthielt.

Die Zellen wurden nach 24, 48 und 72 Stunden geerntet und die Gesamt-RNA mit dem RNeasy-Mini-Kit von Qiagen extrahiert. 10 µg Gesamt-RNA jeder Probe wurden auf einem 1%igen Agarose-Formaldehyd-Gel elektrophoretisch aufgetrennt, auf eine Nylon-Membran geblottet und mit 5'-α^{32}P-dCTP random-markierten, spezifischen Sonden zuerst gegen MDR1 und nach dem Strippen des Blots gegen GAPDH als interne Kontrolle hybridisiert und auf Röntgenfilmen exponiert.

Die Röntgenfilme wurden digitalisiert (Image Master, VDS Pharmacia) und mit der Image-Quant-Software quantifiziert. Dabei wurde ein Abgleich der MDR1-spezifischen Banden mit den entsprechenden GAPDH-Banden durchgeführt.

Ergebnisse:

Die Fig. 25 und 26 zeigen Northern-Blots (Fig. 25a, 26a) mit quantitativer Auswertung der MDR1-spezifischen Banden nach Abgleich mit den entsprechenden GAPDH-Werten (Fig. 25b, 26b). Es konnte eine Reduktion der MDR1-mRNA um bis zu 55 % im Vergleich zur MOCK-Transfektion und um bis zu 45 % im Vergleich zur unspezifischen Kontroll-Transfektion beobachtet werden. Nach 48 h ist eine signifikante Reduktion des MDR1-mRNA-Niveaus mit den als R1, R2, R3 (Tabelle 4) bezeichneten dsRNA-Konstrukten erreicht worden. Mit den R4-dsRNA-Konstrukten wurde nach 48 h keine signifikante Reduktion gegenüber den Kontrollen beobachtet (Fig. 26a und 26b).
Nach 74 h war eine deutlich stärkere Reduktion des MDR1-mRNA-Levels mit R1, R2 und R3 gegenüber den Kontrollen im Vergleich zu den 48 h-Werten zu beobachten (Fig. 25a und 25b).

Mit R4 konnte konnte zu diesem Zeitpunkt ebenfalls eine siginifikante Verringerung des MDR1-mRNA-Niveaus erzielt werden. Somit reduzieren die Konstrukte mit einem 2nt-Überhang am 3´-Ende des Antisinnstrangs und einem doppelsträngigen Bereich aus 22 Nukleotidpaaren, relativ unabhängig von dem jeweiligen zum MDR1-Gen homologen Sequenzbereich (nach 48 h; Fig. 26b) das MDR1-mRNA-Level effizienter als die Konstrukte mit mit 2nt-Überhängen an den 3´-Enden beider Stränge (Antisinn- und Sinnstrang) und einem Doppelstrangbereich von 19 Nukleotidpaaren. Die Ergebnisse bekräftigen damit die in Ausführungsbeispiel IV beschriebene Inhibition der EGFR-Genexpression durch spezifische dsRNAs nach Transfektion in U-87 MG-Zellen.

Die Transfektionseffizienz wurde in einem getrennten Experiment mit Hilfe eines Texas-Red-markierten DNA-Oligonukleotids (TexRed-A$(GATC)_5$T; ebenfalls 175 nM transfiziert) ermittelt (Fig. 27a, 27b; 400fache Vergrößerung, 48h nach Transfektion). Sie betrug etwa 50% auf der Grundlage der rot fluoreszierenden Zellen im Vergleich zur Gesamtzellzahl. Berücksichtigt man die Transfektionsrate der Zellen von etwa 50%, so legt die beobachtete Verringerung des MDR1-mRNA-Niveaus um ca. 45-55% liegt (verglichen mit den Kontrollen), den Schluss nahe, dass in allen Zellen, die mit spezifischer dsRNA erfolgreich transfiziert werden konnten, die MDR1-mRNA nahezu vollständig und spezifisch abgebaut wurde.

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47. Verwendung nach einem der Ansprüche 41 bis 47, wobei die dsRNA I und/oder die dsRNA II eine Länge von weniger als 25, vorzugsweise 19 bis 23, aufeinander folgenden Nukleotidpaaren aufweist/en.

48. Verwendung nach einem der Ansprüche 41 bis 47, wobei der erste (B1) und der zweite Bereich (B2) abschnittsweise überlappen oder aneinander grenzen.

49. Verwendung nach einem der Ansprüche 41 bis 48, wobei der erste (B1) und der zweite Bereich (B2) voneinander beabstandet sind.

50. Verwendung nach einem der Ansprüche 41 bis 49, wobei das Zielgen eine der Sequenzen SQ001 bis SQ140 aufweist.

51. Verwendung nach einem der Ansprüche 41 bis 50, wobei das Zielgen aus der folgenden Gruppe ausgewählt ist: Onkogen, Cytokin-Gen, Id-Protein-Gen, Priongen, Gene von Angiogenese induzierenden Molekülen, von Adhäsions-Molekülen und von Zelloberflächenrezeptoren, Gene von Proteinen,die an metastasierenden und/oder invasiven Prozessen beteiligt sind, Gene von Proteinasen sowie von Apoptose- und Zellzyklusregulierende Molekülen.

52. Verwendung nach einem der Ansprüche 41 bis 51, wobei das Zielgen das MRD1-Gens ist.

53. Verwendung nach einem der Ansprüche 41 bis 52, wobei als dsRNA I/II eine der Sequenzen SQ141 -173 bzw. ein aus zwei jeweils zusammengehörenden Antisinn- (as1/2) und Sinnsequenzen (ss1/2) kombiniertes dsRNA-Konstrukt der Sequenzen SQ141 - 173 verwendet wird.

54. Verwendung nach einem der Ansprüche 41 bis 53, wobei die Expression nach dem Prinzip der RNA-Interferenz gehemmt wird.

55. Verwendung nach einem der Ansprüche 41 bis 54, wobei das Zielgen in pathogenen Organismen, vorzugsweise in Plasmodien, exprimiert wird.

56. Verwendung nach einem der Ansprüche 41 bis 55, wobei das Zielgen Bestandteil eines Virus oder Viroids ist.

57. Verwendung nach Anspruch 56, wobei das Virus ein humanpathogenes Virus oder Viroid ist.

58. Verwendung nach Anspruch 56, wobei das Virus oder Viroid ein tier- oder pflanzenpathogenes Virus oder Viroid ist.

59. Verwendung nach einem der Ansprüche 41 bis 58, wobei ungepaarte Nukleotide durch Nukleosidthiophosphate substituiert sind.

60. Verwendung nach einem der Ansprüche 41 bis 59, wobei zumindest ein Ende (E1, E2) der dsRNA modifiziert wird, um einem Abbau in der Zelle oder einer Dissoziation in die Einzelstränge entgegenzuwirken.

61. Verwendung nach einem der Ansprüche 41 bis 60, wobei der durch die komplementären Nukleotidpaare bewirkte Zusammenhalt der doppelsträngigen Struktur durch mindestens eine chemische Verknüpfung erhöht wird.

62. Verwendung nach einem der Ansprüche 41 bis 61, wobei die chemische Verknüpfung durch eine kovalente oder ionische Bindung, eine Wasserstoffbrückenbindung, hydrophobe Wechselwir-

kungen, vorzugsweise van-der-Waals- oder Stapelungswechselwirkungen, oder durch Metall-Ionenkoordination gebildet wird.

63. Verwendung nach einem der Ansprüche 41 bis 62, wobei die chemische Verknüpfung in der Nähe des einen Endes (E1, E2) gebildet ist.

64. Verwendung nach einem der Ansprüche 41 bis 63, wobei die chemische Verknüpfung mittels einer oder mehrerer Verbindungsgruppen gebildet wird, wobei die Verbindungsgruppen vorzugsweise Poly-(oxyphosphinicooxy-1,3-propandiol)- und/oder Oligoethylenglycol-Ketten sind.

65. Verwendung nach einem der Ansprüche 41 bis 64, wobei die chemische Verknüpfung durch anstelle von Nukleotiden benutzte verzweigte Nukleotidanaloga gebildet wird.

66. Verwendung nach einem der Ansprüche 41 bis 65, wobei die chemische Verknüpfung durch Purinanaloga gebildet wird.

67. Verwendung nach einem der Ansprüche 41 bis 66, wobei die chemische Verknüpfung durch Azabenzoleinheiten gebildet wird.

68. Verwendung nach einem der Ansprüche 41 bis 67, wobei zur Herstellung der chemischen Verknüpfung mindestens eine der folgenden Gruppen benutzt wird: Methylenblau; bifunktionelle Gruppen, vorzugsweise Bis-(2-chlorethyl)-amin; N-acetyl-N´-(p-glyoxyl-benzoyl)-cystamin; 4-Thiouracil; Psoralen.

69. Verwendung nach einem der Ansprüche 41 bis 68, wobei die chemische Verknüpfung durch in der Nähe der Enden (E1, E2) des doppelsträngigen Bereichs angebrachte Thiophosphoryl-Gruppen gebildet wird.

70. Verwendung nach einem der Ansprüche 41 bis 69, wobei die chemische Verknüpfung durch in der Nähe der Enden (E1, E2) befindliche Tripelhelix-Bindungen hergestellt wird.

71. Verwendung nach einem der Ansprüche 41 bis 70, wobei die dsRNA I/II in micellare Strukturen, vorteilhafterweise in Liposomen, eingeschlossen wird.

72. Verwendung nach einem der Ansprüche 41 bis 71, wobei die dsRNA I/II an mindestens ein von einem Virus stammendes, davon abgeleitetes oder ein synthetisch hergestelltes virales Hüllprotein gebunden, damit assoziiert oder davon umgeben wird/werden.

73. Verwendung nach einem der Ansprüche 41 bis 72, wobei das Hüllprotein vom Polyomavirus abgeleitet ist.

74. Verwendung nach einem der Ansprüche 41 bis 73, wobei das Hüllprotein das Virus-Protein 1 (VP1) und/oder das Virus-Protein 2 (VP2) des Polyomavirus enthält.

75. Verwendung nach einem der Ansprüche 41 bis 74, wobei bei Bildung eines Kapsids oder kapsidartigen Gebildes aus dem Hüllprotein die eine Seite zum Inneren des Kapsids oder kapsidartigen Gebildes gewandt ist.

76. Verwendung nach einem der Ansprüche 41 bis 75, wobei der eine Strang (as1, as2) der dsRNA I/II zum primären oder prozessierten RNA-Transkript des Zielgens komplementär ist.

77. Verwendung nach einem der Ansprüche 41 bis 76, wobei die Zelle eine Vertebratenzelle oder eine menschliche Zelle ist.

78. Verwendung nach einem der Ansprüche 41 bis 77, wobei die dsRNA I/II in einer Menge von höchstens 5 mg je Kilogramm Körpergewicht pro Tag einem Säugetier, vorzugsweise einem Menschen, verabreicht wird.

79. Verwendung nach einem der Ansprüche 41 bis 78, wobei die dsRNA I/II zur Applikation in eine Pufferlösung aufgenommen ist.

80. Verwendung nach einem der Ansprüche 41 bis 79, wobei die dsRNA I/II oral oder mittels Injektion oder Infusion intravenös, intratumoral, inhalativ, intraperitoneal verabreicht wird.

81. Medikament zur Hemmung der Expression eines Zielgens in einer Zelle enthaltend eine doppelsträngige Ribonukleinsäure (dsRNA I) in einer zur Hemmung der Expression des Zielgens ausreichenden Menge,

wobei die dsRNA I eine doppelsträngige aus höchstens 49 aufeinander folgenden Nukleotidpaaren gebildete Struktur aufweist,

und wobei ein Strang (as1) oder zumindest ein Abschnitt des einen Strangs (as1) der doppelsträngigen Struktur komplementär zum Zielgen ist,

und wobei die dsRNA I zumindest am einen Ende (E1, E2) einen aus 1 bis 4 Nukleotiden gebildeten Überhang aufweist.

82. Medikament nach Anspruch 81, wobei die dsRNA I den Überhang am 3'-Ende des einen Strangs (as1) und/oder am 3'-Ende des anderen Strangs (ss1) aufweist.

126. Verfahren nach einem der vorhergehenden Ansprüche, wobei zumindest eine entsprechend der dsRNA I nach einem der vorhergehenden Ansprüche ausgebildete weitere doppelsträngige Ribonukleinesäure (dsRNA II) in die Zelle eingeführt wird, wobei der eine Strang (as1) oder zumindest ein Abschnitt des einen Strangs (as1) der dsRNA I komplementär zu einem ersten Bereich (B1) des Zielgens ist, und wobei ein weiterer Strang (as2) oder zumindest ein Abschnitt des weiteren Strangs (as2) der dsRNA II komplementär zu einem zweiten Bereich (B2) des Zielgens ist.

127. Verfahren nach einem der vorhergehenden Ansprüche, wobei die dsRNA I und/oder die dsRNA II eine Länge von weniger als 25, vorzugsweis 19 bis 23, aufeinander folgenden Nukleotidpaaren aufweist/en.

128. Verfahren nach einem der vorhergehenden Ansprüche, wobei der erste (B1) und der zweite Bereich (B2) abschnittsweise überlappen oder aneinander grenzen.

129. Verfahren nach einem der vorhergehenden Ansprüche, wobei der erste (B1) und der zweite Bereich (B2) voneinander beabstandet sind.

130. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Zielgen eine der Sequenzen SQ001 bis SQ140 aufweist.

131. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Zielgen aus der folgenden Gruppe ausgewählt ist: Onkogen, Cytokin-Gen, Id-Protein-Gen, Priongen, Gene von Angiogenese induzierenden Molekülen, von Adhäsions-Molekülen und von Zelloberflächenrezeptoren, Gene von Proteinen, die an metastasierenden und/oder invasiven Prozessen beteiligt sind, Ge-

ne von Proteinasen sowie Apoptose- und Zellzyklus-regulierenden Molekülen.

132. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Zielgen das MDR1-Gens ist.

133. Verfahren nach einem der vorhergehenden Ansprüche, wobei als dsRNA I/II eine der Sequenzen SQ141 -173 bzw. ein aus zwei jeweils zusammengehörenden Antisinn- (as1/2) und Sinnsequenzen (ss1/2) kombiniertes dsRNA-Konstrukt der Sequenzen SQ141 - 173 verwendet wird.

134. Verfahren nach einem der vorhergehenden Ansprüche, wobei die Expression nach dem Prinzip der RNA-Interferenz gehemmt wird.

135. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Zielgen in pathogenen Organismen, vorzugsweise in Plasmodien, exprimiert wird.

136. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Zielgen Bestandteil eines Virus oder Viroids ist.

137. Verfahren nach Anspruch 16, wobei das Virus ein humanpathogenes Virus oder Viroid ist.

138. Verfahren nach Anspruch 16, wobei das Virus oder Viroid ein tier- oder pflanzenpathogenes Virus oder Viroid ist.

139. Verfahren nach einem der vorhergehenden Ansprüche, wobei ungepaarte Nukleotide durch Nukleosidthiophosphate substituiert sind.

140. Verfahren nach einem der vorhergehenden Ansprüche, wobei zumindest ein Ende (E1, E2) der dsRNA I/II modifiziert wird, um einem Abbau in der Zelle oder einer Dissoziation in die Einzelstränge entgegenzuwirken.

141. Verfahren nach einem der vorhergehenden Ansprüche, wobei der durch die komplementären Nukleotidpaare bewirkte Zusammenhalt der doppelsträngigen Struktur durch mindestens eine chemische Verknüpfung erhöht wird.

142. Verfahren nach einem der vorhergehenden Ansprüche, wobei die chemische Verknüpfung durch eine kovalente oder ionische Bindung, eine Wasserstoffbrückenbindung, hydrophobe Wechselwirkungen, vorzugsweise van-der-Waals- oder Stapelungswechselwirkungen, oder durch Metall-Ionenkoordination gebildet wird.

143. Verfahren nach einem der vorhergehenden Ansprüche, wobei die chemische Verknüpfung in der Nähe des einen Endes (E1, E2) gebildet ist.

144. Verfahren nach einem der vorhergehenden Ansprüche, wobei die chemische Verknüpfung mittels einer oder mehrerer Verbindungsgruppen gebildet wird, wobei die Verbindungsgruppen vorzugsweise Poly-(oxyphosphinicooxy-1,3-propandiol)- und/oder Oligoethylenglycol-Ketten sind.

145. Verfahren nach einem der vorhergehenden Ansprüche, wobei die chemische Verknüpfung durch anstelle von Nukleotiden benutzte verzweigte Nukleotidanaloga gebildet wird.

146. Verfahren nach einem der vorhergehenden Ansprüche, wobei die chemische Verknüpfung durch Purinanaloga gebildet wird.

147. Verfahren nach einem der vorhergehenden Ansprüche, wobei die chemische Verknüpfung durch Azabenzoleinheiten gebildet wird.

148. Verfahren nach einem der vorhergehenden Ansprüche, wobei zur Herstellung der chemischen Verknüpfung mindestens eine der folgenden Gruppen benutzt wird: Methylenblau; bifunktionelle Gruppen, vorzugsweise Bis-(2-chlorethyl)-amin; N-acetyl-N´-(p-glyoxyl-benzoyl)-cystamin; 4-Thiouracil; Psoralen.

149. Verfahren nach einem der vorhergehenden Ansprüche, wobei die chemische Verknüpfung durch in der Nähe der Enden (E1, E2) des doppelsträngigen Bereichs angebrachte Thiophosphoryl-Gruppen gebildet wird.

150. Verfahren nach einem der vorhergehenden Ansprüche, wobei die chemische Verknüpfung durch in der Nähe der Enden (E1, E2) befindliche Tripelhelix-Bindungen hergestellt wird.

151. Verfahren nach einem der vorhergehenden Ansprüche, wobei die dsRNA I/II in micellare Strukturen, vorteilhafterweise in Liposomen, eingeschlossen wird.

152. Verfahren nach einem der vorhergehenden Ansprüche, wobei die dsRNA I/II an mindestens ein von einem Virus stammendes, davon abgeleitetes oder ein synthetisch hergestelltes virales Hüllprotein gebunden, damit assoziiert oder davon umgeben wird/werden.

153. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Hüllprotein vom Polyomavirus abgeleitet ist.

154. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Hüllprotein das Virus-Protein 1 (VP1) und/oder das Virus-Protein 2 (VP2) des Polyomavirus enthält.

155. Verfahren nach einem der vorhergehenden Ansprüche, wobei bei Bildung eines Kapsids oder kapsidartigen Gebildes aus dem Hüllprotein die eine Seite zum Inneren des Kapsids oder kapsidartigen Gebildes gewandt ist.

156. Verfahren nach einem der vorhergehenden Ansprüche, wobei der eine Strang (as1, as2) der dsRNA I/II zum primären oder prozessierten RNA-Transkript des Zielgens komplementär ist.

157. Verfahren nach einem der vorhergehenden Ansprüche, wobei die Zelle eine Vertebratenzelle oder eine menschliche Zelle ist.

158. Verfahren nach einem der vorhergehenden Ansprüche, wobei

kungen, vorzugsweise van-der-Waals- oder Stapelungswechselwirkungen, oder durch Metall-Ionenkoordination gebildet wird.

183. Verwendung nach einem der Ansprüche 41 bis 62, wobei die chemische Verknüpfung in der Nähe des einen Endes (E1, E2) gebildet ist.

184. Verwendung nach einem der Ansprüche 41 bis 63, wobei die chemische Verknüpfung mittels einer oder mehrerer Verbindungsgruppen gebildet wird, wobei die Verbindungsgruppen vorzugsweise Poly-(oxyphosphinicooxy-1,3-propandiol)- und/oder Oligoethylenglycol-Ketten sind.

185. Verwendung nach einem der Ansprüche 41 bis 64, wobei die chemische Verknüpfung durch anstelle von Nukleotiden benutzte verzweigte Nukleotidanaloga gebildet wird.

186. Verwendung nach einem der Ansprüche 41 bis 65, wobei die chemische Verknüpfung durch Purinanaloga gebildet wird.

187. Verwendung nach einem der Ansprüche 41 bis 66, wobei die chemische Verknüpfung durch Azabenzoleinheiten gebildet wird.

188. Verwendung nach einem der Ansprüche 41 bis 67, wobei zur Herstellung der chemischen Verknüpfung mindestens eine der folgenden Gruppen benutzt wird: Methylenblau; bifunktionelle Gruppen, vorzugsweise Bis-(2-chlorethyl)-amin; N-acetyl-N´-(p-glyoxyl-benzoyl)-cystamin; 4-Thiouracil; Psoralen.

189. Verwendung nach einem der Ansprüche 41 bis 68, wobei die chemische Verknüpfung durch in der Nähe der Enden (E1, E2) des doppelsträngigen Bereichs angebrachte Thiophosphoryl-Gruppen gebildet wird.

190. Verwendung nach einem der Ansprüche 41 bis 69, wobei die chemische Verknüpfung durch in der Nähe der Enden (E1, E2) befindliche Tripelhelix-Bindungen hergestellt wird.

191. Verwendung nach einem der Ansprüche 41 bis 70, wobei die dsRNA I/II in micellare Strukturen, vorteilhafterweise in Liposomen, eingeschlossen wird.

192. Verwendung nach einem der Ansprüche 41 bis 71, wobei die dsRNA I/II an mindestens ein von einem Virus stammendes, davon abgeleitetes oder ein synthetisch hergestelltes virales Hüllprotein gebunden, damit assoziiert oder davon umgeben wird/werden.

193. Verwendung nach einem der Ansprüche 41 bis 72, wobei das Hüllprotein vom Polyomavirus abgeleitet ist.

194. Verwendung nach einem der Ansprüche 41 bis 73, wobei das Hüllprotein das Virus-Protein 1 (VP1) und/oder das Virus-Protein 2 (VP2) des Polyomavirus enthält.

195. Verwendung nach einem der Ansprüche 41 bis 74, wobei bei Bildung eines Kapsids oder kapsidartigen Gebildes aus dem Hüllprotein die eine Seite zum Inneren des Kapsids oder kapsidartigen Gebildes gewandt ist.

196. Verwendung nach einem der Ansprüche 41 bis 75, wobei der eine Strang (as1, as2) der dsRNA I/II zum primären oder prozessierten RNA-Transkript des Zielgens komplementär ist.

197. Verwendung nach einem der Ansprüche 41 bis 76, wobei die Zelle eine Vertebratenzelle oder eine menschliche Zelle ist.

198. Verwendung nach einem der Ansprüche 41 bis 77, wobei die dsRNA I/II in einer Menge von höchstens 5 mg je Kilogramm Körpergewicht pro Tag einem Säugetier, vorzugsweise einem Menschen, verabreicht wird.

199. Verwendung nach einem der Ansprüche 41 bis 78, wobei die dsRNA I/II zur Applikation in eine Pufferlösung aufgenommen ist.

200. Verwendung nach einem der Ansprüche 41 bis 79, wobei die dsRNA I/II oral oder mittels Injektion oder Infusion intravenös, intratumoral, inhalativ, intraperitoneal verabreicht wird.

201. Medikament zur Hemmung der Expression eines Zielgens in einer Zelle enthaltend eine doppelsträngige Ribonukleinsäure (dsRNA I) in einer zur Hemmung der Expression des Zielgens ausreichenden Menge,

wobei die dsRNA I eine doppelsträngige aus höchstens 49 aufeinander folgenden Nukleotidpaaren gebildete Struktur aufweist,

und wobei ein Strang (as1) oder zumindest ein Abschnitt des einen Strangs (as1) der doppelsträngigen Struktur komplementär zum Zielgen ist,

und wobei die dsRNA I zumindest am einen Ende (E1, E2) einen aus 1 bis 4 Nukleotiden gebildeten Überhang aufweist.

202. Medikament nach Anspruch 81, wobei die dsRNA I den Überhang am 3'-Ende des einen Strangs (as1) und/oder am 3'-Ende des anderen Strangs (ss1) aufweist.

Cytokin-Gen, Id-Protein-Gen, Priongen, Gene von Angiogenese induzierenden Molekülen, von Adhäsions-Molekülen und von Zelloberflächenrezeptoren, Gene von Proteinen, die an metastasierenden und/oder invasiven Prozessen beteiligt sind, Gene von Proteinasen sowie von Apoptose- und Zellzyklusregulierende Molekülen.

211. Medikament nach einem der Ansprüche 81 bis 90, wobei das Zielgen das MRD1-Gen ist.

212. Medikament nach einem der Ansprüche 81 bis 91, wobei als dsRNA eine der Sequenzen SQ141 -173 bzw. ein aus zwei jeweils zusammengehörenden Antisinn- (as1/2) und Sinnsequenzen (ss1/2) kombiniertes dsRNA-Konstrukt der Sequenzen SQ141 - 173 verwendet wird.

213. Medikament nach einem der Ansprüche 81 bis 92, wobei die Expression nach dem Prinzip der RNA-Interferenz gehemmt wird.

214. Medikament nach einem der Ansprüche 81 bis 93, wobei das Zielgen in pathogenen Organismen, vorzugsweise in Plasmodien, exprimierbar ist.

215. Medikament nach einem der Ansprüche 81 bis 94, wobei das Zielgen Bestandteil eines Virus oder Viroids ist.

216. Medikament nach Anspruch 95, wobei das Virus ein humanpathogenes Virus oder Viroid ist.

217. Medikament nach Anspruch 95, wobei das Virus oder Viroid ein tier- oder pflanzenpathogenes Virus oder Viroid ist.

218. Medikament nach einem der Ansprüche 81 bis 97, wobei ungepaarte Nukleotide durch Nukleosidthiophosphate substituiert sind.

219. Medikament nach einem der Ansprüche 81 bis 98, wobei zumindest ein Ende (E1, E2) der dsRNA modifiziert ist, um einem Abbau in der Zelle oder einer Dissoziation in die Einzelstränge entgegenzuwirken.

220. Medikament nach einem der Ansprüche 81 bis 99, wobei der durch die komplementären Nukleotidpaare bewirkte Zusammenhalt der doppelsträngigen Struktur durch mindestens eine chemische Verknüpfung erhöht ist.

221. Medikament nach einem der Ansprüche 81 bis 100, wobei die chemische Verknüpfung durch eine kovalente oder ionische Bindung, eine Wasserstoffbrückenbindung, hydrophobe Wechselwirkungen, vorzugsweise van-der-Waals- oder Stapelungswechselwirkungen, oder durch Metall-Ionenkoordination gebildet ist.

222. Medikament nach einem der Ansprüche 81 bis 101, wobei die chemische Verknüpfung in der Nähe des einen Endes (E1, E2) gebildet ist.

223. Medikament nach einem der Ansprüche 81 bis 102, wobei die chemische Verknüpfung mittels einer oder mehrerer Verbindungsgruppen gebildet wird, wobei die Verbindungsgruppen vorzugsweise Poly-(oxyphosphinicooxy-1,3-propandiol)- und/oder Oligoethylenglycol-Ketten sind.

224. Medikament nach einem der Ansprüche 81 bis 103, wobei die chemische Verknüpfung durch anstelle von Nukleotiden benutzte verzweigte Nukleotidanaloga gebildet ist.

225. Medikament nach einem der Ansprüche 81 bis 104, wobei die chemische Verknüpfung durch Purinanaloga gebildet ist.

226. Medikament nach einem der Ansprüche 81 bis 105, wobei die chemische Verknüpfung durch Azabenzoleinheiten gebildet ist.

227. Medikament nach einem der Ansprüche 81 bis 106, wobei zur Herstellung der chemischen Verknüpfung mindestens eine der folgenden Gruppen benutzt wird: Methylenblau; bifunktionelle Gruppen, vorzugsweise Bis-(2-chlorethyl)-amin; N-acetyl-N´-(p-glyoxyl-benzoyl)-cystamin; 4-Thiouracil; Psoralen.

228. Medikament nach einem der Ansprüche 81 bis 107, wobei die chemische Verknüpfung durch in der Nähe der Enden (E1, E2) des doppelsträngigen Bereichs angebrachte Thiophosphoryl-Gruppen gebildet ist.

229. Medikament nach einem der Ansprüche 81 bis 108, wobei die chemische Verknüpfung durch in der Nähe der Enden (E1, E2) befindliche Tripelhelix-Bindungen hergestellt ist.

230. Medikament nach einem der Ansprüche 81 bis 109, wobei die dsRNA I/II in micellare Strukturen, vorteilhafterweise in Liposomen, eingeschlossen ist.

231. Medikament nach einem der Ansprüche 81 bis 110, wobei die dsRNA I an mindestens ein von einem Virus stammendes, davon abgeleitetes oder ein synthetisch hergestelltes virales Hüllprotein gebunden, damit assoziiert oder davon umgeben ist/sind.

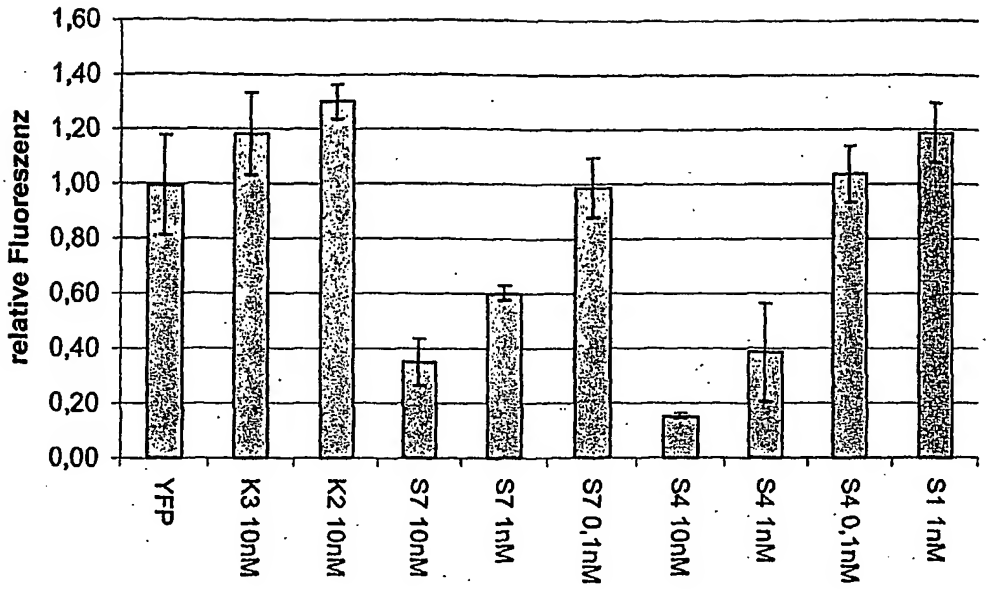
relative Fluoreszenz
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
YFP
K3 10nM
K2 10nM
S7 10nM
S7 1nM
S7 0,1nM
S4 10nM
S4 1nM
S4 0,1nM
S1 1nM

Fig. 3

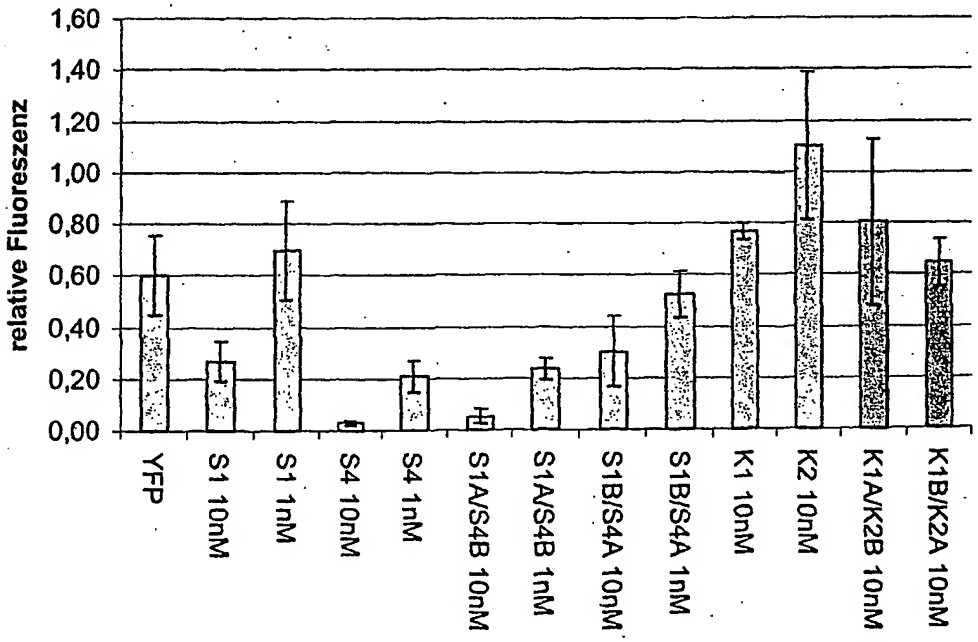
relative Fluoreszenz
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
YFP
S1 10nM
S1 1nM
S4 10nM
S4 1nM
S1A/S4B 10nM
S1A/S4B 1nM
S1B/S4A 10nM
S1B/S4A 1nM
K1 10nM
K2 10nM
K1A/K2B 10nM
K1B/K2A 10nM

Fig. 4

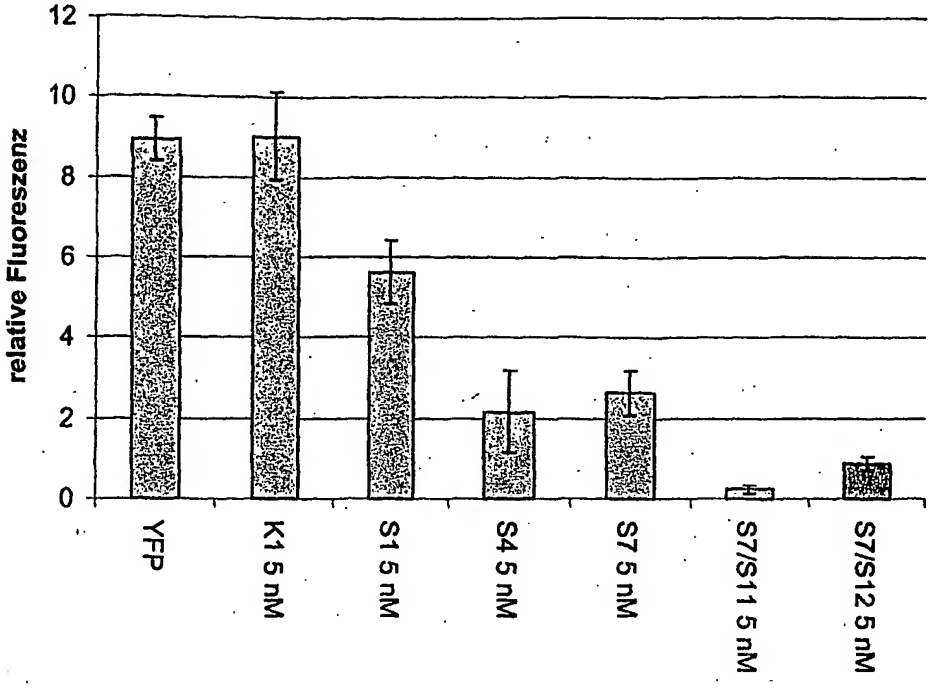
relative Fluoreszenz
12
10
8
6
4
2
0
YFP
K1 5 nM
S1 5 nM
S4 5 nM
S7 5 nM
S7/S11 5 nM
S7/S12 5 nM

Fig. 5

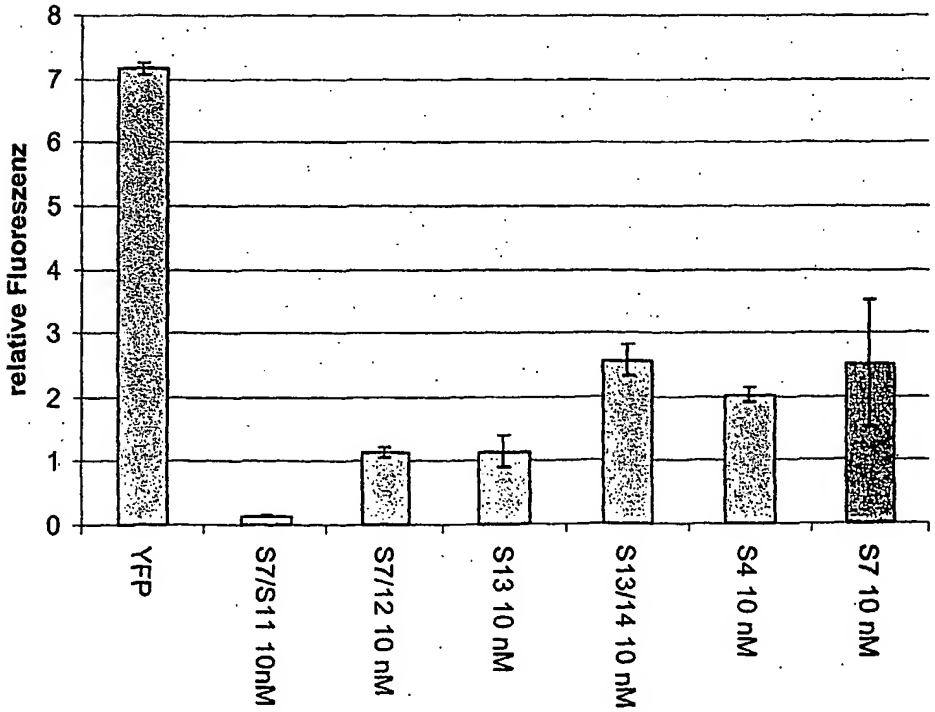
relative Fluoreszenz
8
7
6
5
4
3
2
1
0
YFP
S7/S11 10nM
S7/12 10 nM
S13 10 nM
S13/14 10 nM
S4 10 nM
S7 10 nM

Fig. 6

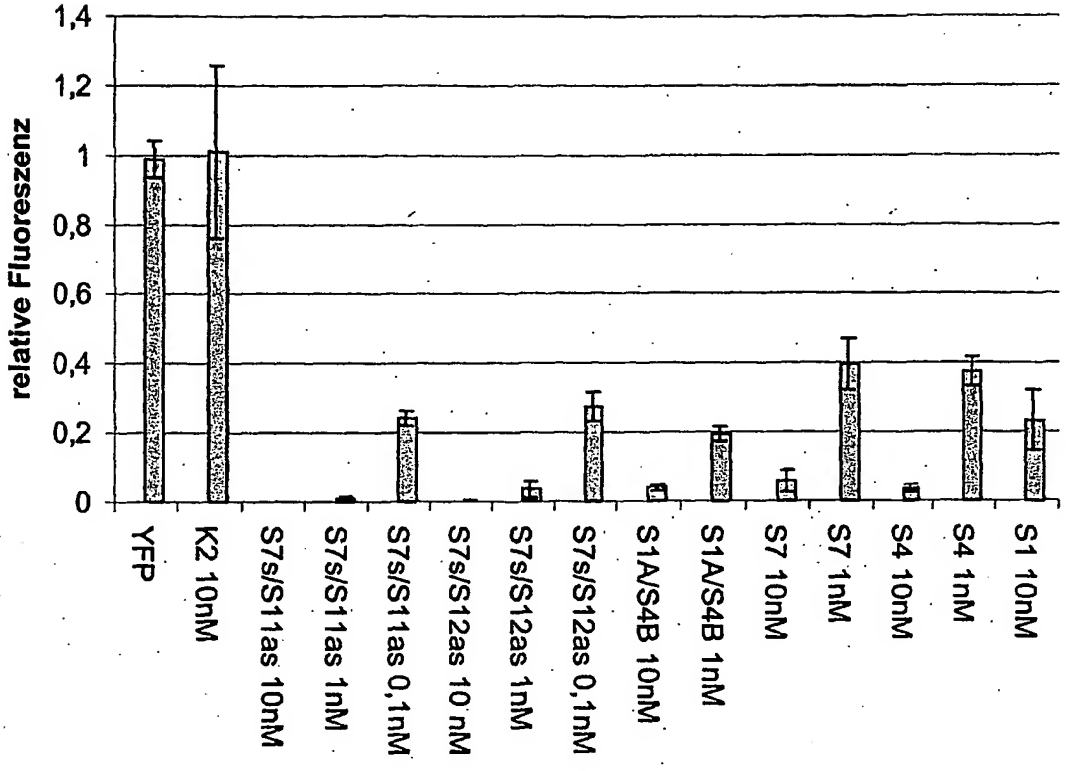
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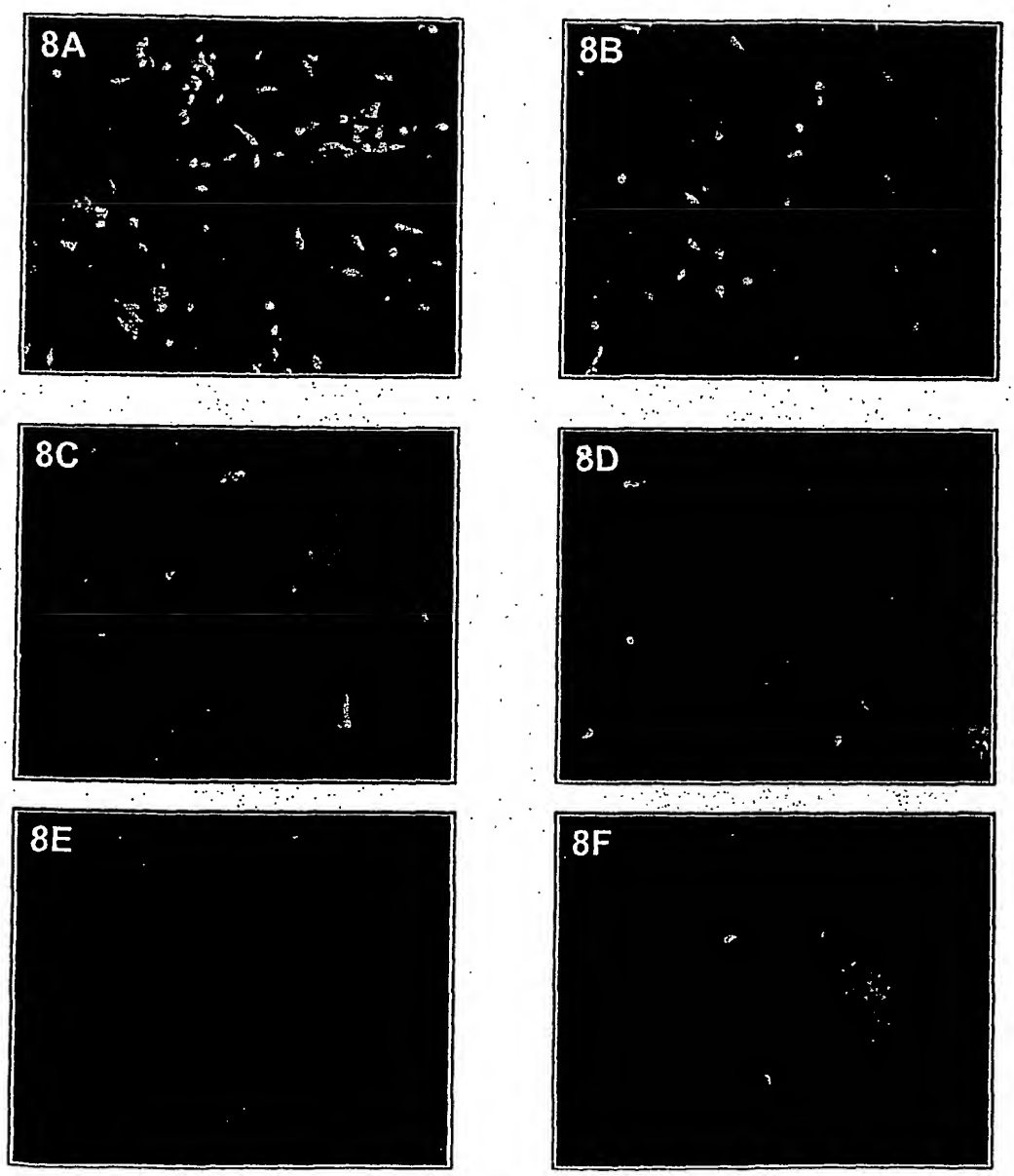
Fig. 7



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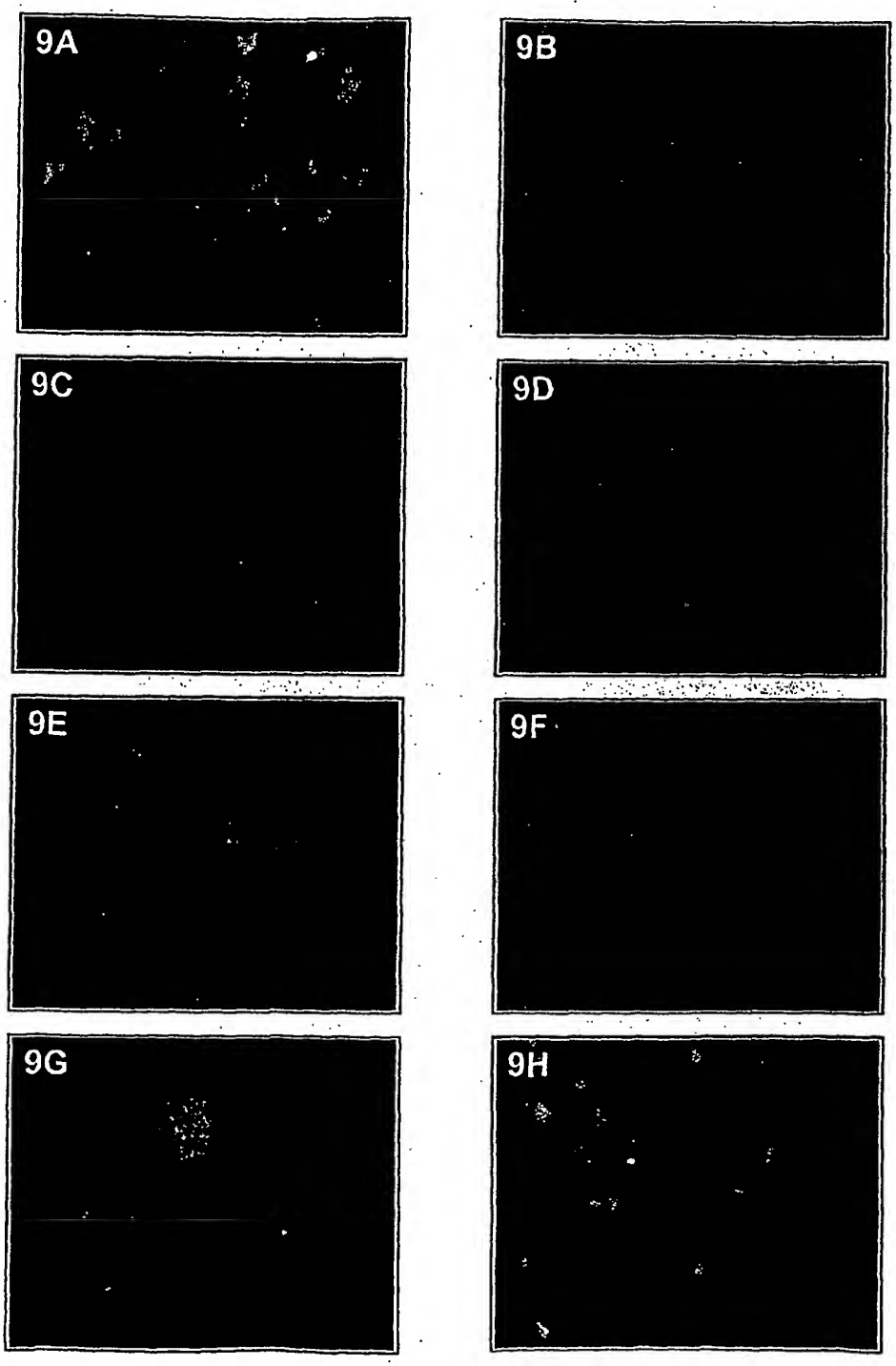

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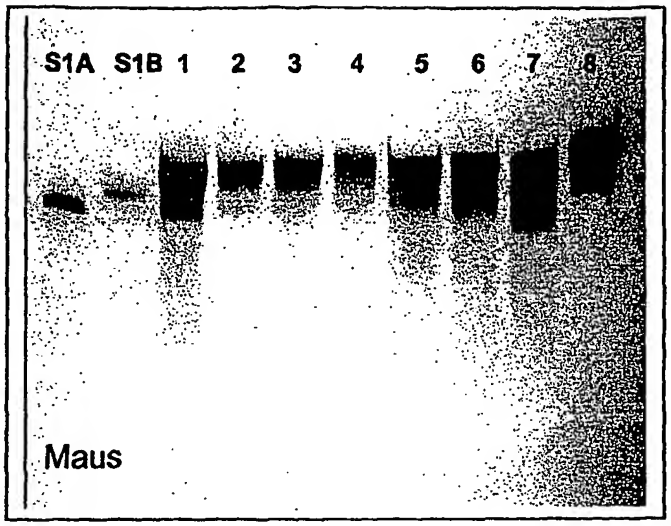

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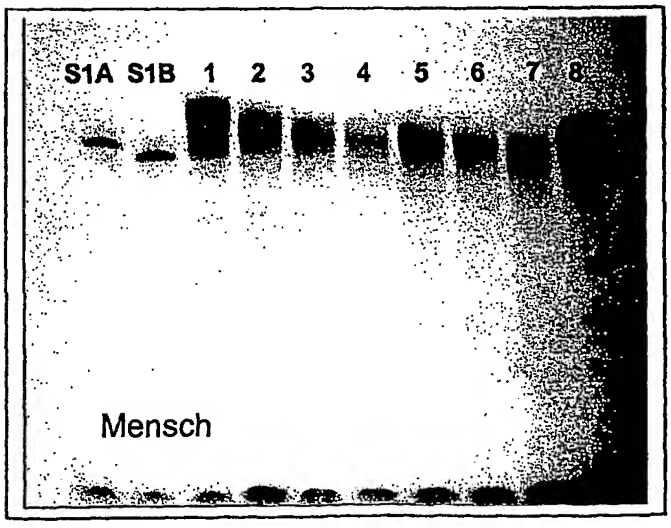
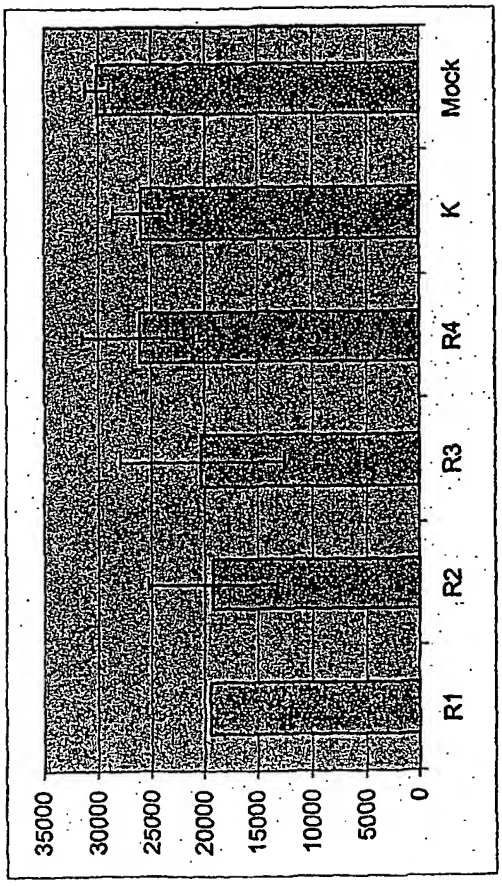
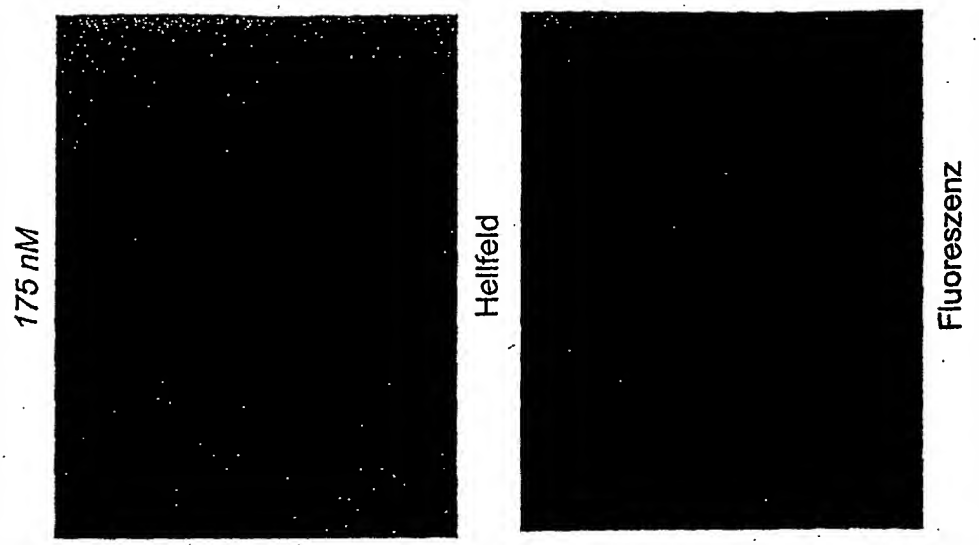


Fig. 11

Fig. 26b

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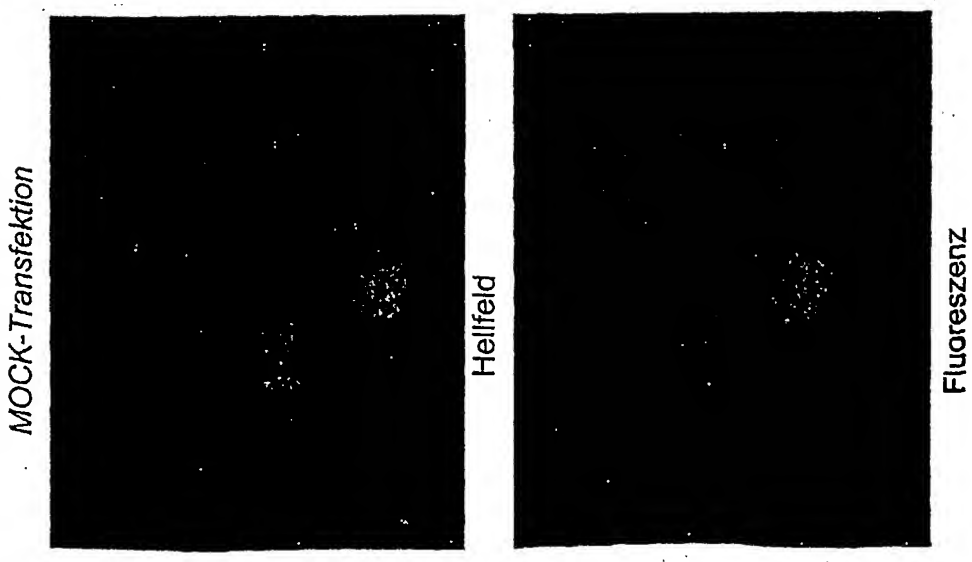
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Fig. 27

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ccagcattgt tttctcttat aattggcttt gcttttggag ccttatactg gaagaagaga 720
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<220>
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<400> 155
gaugaggauc guuucgcaug a 21

<210> 156
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<213> Künstliche Sequenz

<220>
<223> Beschreibung der künstlichen Sequenz: antisense-Strang (K3B) einer dsRNA, die komplementär zur 5`-UTR der Neomycin-Sequenz ist

<400> 156
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<210> 157
<211> 24
<212> RNA
<213> Künstliche Sequenz

<220>
<223> Beschreibung der künstlichen Sequenz: sense-Strang (K2A) einer dsRNA, die homolog zur 5`-UTR der Neomycin-Sequenz ist

<400> 157
acaggaugag gaucguuucg caug 24

<210> 158
<211> 24
<212> RNA
<213> Künstliche Sequenz

<220>
<223> Beschreibung der künstlichen Sequenz: antisense-Strang (K2B) einer dsRNA, die komplementär zur 5`-UTR der Neomycin-Sequenz ist

<400> 158
ugcgaaacga uccucauccu gucu 24

<210> 159
<211> 24
<212> RNA
<213> Künstliche Sequenz

<220>

<223> Beschreibung der künstlichen Sequenz:
antisense-Strang (S4B) einer dsRNA, die
komplementär zur YFP-bzw. GFP-Sequenz ist

<400> 159
gaagucgugc ugcuucaugu gguc 24

<210> 160
<211> 24
<212> RNA
<213> Künstliche Sequenz

<220>
<223> Beschreibung der künstlichen Sequenz: sense-Strang
(PKC1 A) einer dsRNA, die homolog zur
Proteinkinase C-Sequenz ist

<400> 160
cuucuccgcc ucacaccgcu gcaa 24

<210> 161
<211> 22
<212> RNA
<213> Künstliche Sequenz

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antisense-Strang (PKC2 B) einer dsRNA, die
komplementär zur Proteinkinase C-Sequenz ist

<400> 161
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<210> 162
<211> 21
<212> RNA
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antisense-Strang (S12B) einer dsRNA, die
komplementär zur YFP- bzw. GFP-Sequenz ist

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<210> 163
<211> 23
<212> RNA
<213> Künstliche Sequenz

<220>
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antisense-Strang (S11B) einer dsRNA, die
komplementär zur YFP- bzw. GFP-Sequenz ist

<400> 163
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<210> 164
<211> 20
<212> RNA
<213> Künstliche Sequenz

<220>
<223> Beschreibung der künstlichen Sequenz: sense-Strang (S13A) einer dsRNA, die homolog zur YFP- bzw. GFP-Sequenz ist

<400> 164
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<210> 165
<211> 22
<212> RNA
<213> Künstliche Sequenz

<220>
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<400> 165
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<210> 166
<211> 20
<212> RNA
<213> Künstliche Sequenz

<220>
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<210> 167
<211> 24
<212> RNA
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<220>
<223> Beschreibung der künstlichen Sequenz: sense-Strang (S4A) einer dsRNA, die homolog zur YFP- bzw. GFP-Sequenz ist

<400> 167
ccacaugaag cagcacgacu ucuu 24

<210> 168
<211> 21
<212> RNA
<213> Künstliche Sequenz

<220>
<223> Beschreibung der künstlichen Sequenz: sense-Strang (ES-7A) einer dsRNA, die homolog zur humanen EGFR-Sequenz ist

<400> 168
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<210> 169
<211> 21
<212> RNA
<213> Künstliche Sequenz

<220>
<223> Beschreibung der künstlichen Sequenz: antisense-Strang (ES-7B) einer dsRNA, die komplementär zur humanen EGFR-Sequenz ist

<400> 169
cuugacaugc ugcgguguuu u 21

<210> 170
<211> 22
<212> RNA
<213> Künstliche Sequenz

<220>
<223> Beschreibung der künstlichen Sequenz: sense-Strang (ES-8A) einer dsRNA, die homolog zur humanen EGFR-Sequenz ist

<400> 170
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<210> 171
<211> 22
<212> RNA
<213> Künstliche Sequenz

<220>
<223> Beschreibung der künstlichen Sequenz: antisense-Strang (ES-8B) einer dsRNA, die komplementär zur humanen EGFR-Sequenz ist

<400> 171
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<210> 172
<211> 22
<212> RNA
<213> Künstliche Sequenz

<220>
<223> Beschreibung der künstlichen Sequenz: sense-Strang (ES-2A) einer dsRNA, die homolog zur humanen EGFR-Sequenz ist

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<210> 173
<211> 24
<212> RNA
<213> Künstliche Sequenz

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<223> Beschreibung der künstlichen Sequenz:
antisense-Strang (ES-5B) einer dsRNA, die
komplementär zur humanen EGFR-Sequenz ist

<400> 173
uugggacagc uuggaucaca cuuu 24

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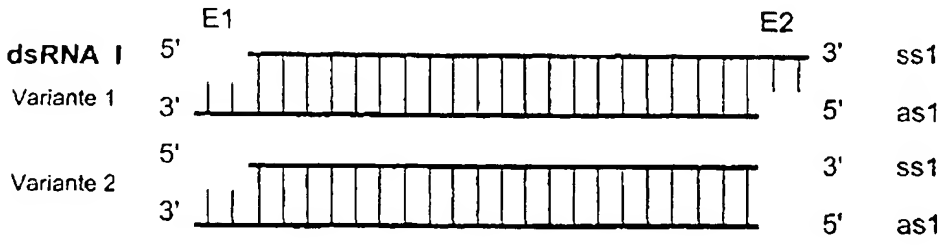
(54) Title: **METHOD FOR INHIBITING THE EXPRESSION OF A TARGET GENE**

(57) Abstract

The invention relates to a method for inhibiting the expression of a target gene in a cell, comprising the following steps: introduction of an amount of at least one dual-stranded ribonucleic acid (dsRNA I) which is sufficient to inhibit the expression of the target gene. The dsRNA I has a dual-stranded structure formed by a maximum of 49 successive nucleotide pairs. One strand (as1) or at least one section of the one strand (as1) of the dual-stranded structure is complementary to the sense strand of the target gene. The dsRNA has an overhang on the end (E1) of dsRNA I formed by 1 – 4 nucleotides.



Variant 1

Variant 2

METHOD FOR INHIBITING THE EXPRESSION OF A TARGET GENE

The invention concerns a method, an application and a medicament for inhibition of expression of a target gene.

Methods to inhibit expression of genes of medical or biotechnological interest by means of a double-strand ribonucleic acid (dsRNA) are known from WO 99/32619 and WO 00/44895. The known methods are highly effective. But there is also a requirement to further increase their efficiency.

The task of the present invention is to eliminate the shortcomings according to the prior art. In particular, a method, an application and a medicament are to be offered, with which even more efficient inhibition of expression of the target gene is attainable.

This task is solved by the features of Claims 1, 41 and 81. Advantageous embodiments are apparent from the features of Claims 2 to 40, 42 to 80 and 82 to 120.

With the features claimed according to the invention, a drastic increase in the effectiveness of inhibition of expression of the target gene in vitro and in vivo is surprisingly achieved. By the special design of the ends of the dsRNA, both their efficiency in mediating the inhibiting effect on expression of the target gene and their stability can be deliberately influenced. By increasing the stability, the effective concentration in the cell is increased.

Target gene according to the invention is understood to mean the DNA strand of the double-strand DNA in the cell that is complementary to a DNA strand, including all transcribed regions that serve for transcription as matrix. The target gene is therefore generally the "sense" strand. The one strand or anti-sense strand (as1) can be complementary to an RNA transcript formed during expression of the target gene or its processing product, for example, an mRNA. Insertion is understood to mean uptake in the cell. Uptake can occur by the cell itself; it also can be mediated by auxiliaries or aids. "Overhang" is understood to mean a terminal single-strand overhang that does not have paired nucleotides, according to Watson and Crick. "Double-strand

structure" is understood to mean a structure, in which the nucleotides of the individual strands are essentially paired according to Watson and Crick. A double-strand structure in the context of the present invention can also have individual mismatches.

According to a particularly advantageous embodiment, the dsRNA I has the overhang on the 3' end of one strand or the anti-sense strand as1 and/or on the 3' end of the other end or sense strand ss1. The dsRNA I can also be formed smoothly on one end. In this case, the smooth end is advantageously situated on the side of dsRNA I that has the 5' end of one strand (anti-sense strand; as1). In this form, the dsRNA I, on the one hand, has very good efficiency and, on the other hand, high stability in a living organism. The overall in vivo efficiency is excellent. The overhang is expediently formed from 1 to 4 nucleotides, preferably from 1 or 2 nucleotides.

According to another embodying feature, the efficiency of the method can be further increased if at least one additional dsRNA II, formed according to the dsRNA I according to the invention, is inserted into the cell, in which the one strand or at least a section of one strand of the double-strand structure of dsRNA I is complementary to a first region of the sense strand of the target gene, and in which an additional strand or at least a section of the additional strand of the double-strand structure of the additional dsRNA II is complementary to a second region of sense strand of the target gene. Inhibition of expression of the target gene is significantly increased in this case. The first and second region can overlap in sections, abut each other, or also be spaced from each other.

It has also proven advantageous if the dsRNA I and/or the additional dsRNA II have a length of less than 25 consecutive nucleotide pairs. A length in the range between 19 and 23 nucleotide pairs has proven to be particularly effective. The efficiency can be further increased if single-strand overhangs of 1 to 4 nucleotides are present on the double strands preferably formed from 19 to 23 nucleotide pairs.

The target gene, according to an additional embodying feature, can have the sequences SQ001 to SQ140, given in the enclosed sequence protocol. It can also be chosen from the following groups: oncogene, cytokine gene, id-protein gene, prion gene, genes for expression of

angiogenesis-inducing molecules, adhesion molecules and cell surface receptors. genes of proteins that participate in metastasizing and/or invasive processes, genes of proteinases, as well as apoptosis and cell cycle-regulating molecules, as well as genes for expression of the EGF receptors. The target gene can be the MDR1 gene, in particular. In this context, one of the existing sequences SQ141-173 or a combined dsRNA I/II from antisense (as) and sense sequences (ss) that go together can be used.

According to an additional advantageous embodying feature, expression is inhibited according to the principle of RNA interference.

The target gene is expediently expressed in pathogenic organisms, preferably in plasmodia. It can be a component of a virus or viroid, especially a human pathogenic virus or viroid. The virus or viroid can also be an animal or plant pathogenic virus or viroid.

According to another embodying feature, it is prescribed that the unpaired nucleotides be substituted by nucleoside thiophosphates.

At least one end of the dsRNA I/II can be modified, in order to counteract degradation in the cell or dissociation in the individual strand. Advantageously, cohesion caused by the complementary nucleotide pairs of the double-strand structure is increased by at least one chemical length. The chemical length can be formed by a covalent or ionic bond, a hydrogen bridge bond, hydrophobic interactions, preferably van-der-Waals or stacking interactions, or by metal ion coordination. It has also proven expedient and to increase stability if the chemical link is formed in the vicinity of one end. Additional advantageous embodiments with respect to chemical linking can be deduced from the features of Claims 24 to 30, without requiring a further explanation for this.

The dsRNA I/II can be incorporated particularly easily in the cell if it is enclosed in micellar structures, advantageously in liposomes. It has also proven advantageous for transport of dsRNA I/II into the cell that it is bonded to at least a viral sheath protein, originating from a virus, derived from a virus or synthetically produced, associated with the sheath protein or

enclosed by it. The sheath protein can be derived from polyoma virus. The sheath protein can contain, in particular, the virus protein 1 and/or the virus protein 2 of polyoma virus. According to another embodiment, it is prescribed that, during formation of a capsid or capsid-like structure from the sheath protein, one side face the interior of the capsid or capsid-like structure. It is also advantageous that the one strand of dsRNA I/II (as1/2) is complementary to the primary or processed RNA transcript of the target gene. The cell can be a vertebrate cell or a human cell.

It has also been found that the dsRNA I/II can advantageously be administered already in an amount of, at most, 5 mg/kg of body weight per day to a mammal, preferably a human. Even in this low dose, an excellent efficiency is achieved.

It has surprisingly been found that the dsRNA I/II can be taken up in a buffer solution for administration and then administered orally or by injection or infusion intravenously, intratumorally, by inhalation or intraperitoneally.

The use of a double-strand ribonucleic acid (dsRNA I) to inhibit expression of a target gene in the cell is also proposed according to the invention, in which the dsRNA I has a double-strand structure formed from, at most, 49 consecutive nucleotide pairs, and in which one strand (anti-sense strand; as1) or at least a section of one strand (as1) of the double-strand structure is complementary to the sense strand of the target gene, and in which the dsRNA I has an overhang formed from 1 to 4 nucleotides on at least one end.

According to an additional stipulation of the invention, a medicament for inhibiting the expression of a target gene in a cell is proposed, containing a double-strand ribonucleic acid (dsRNA I) in an amount sufficient to inhibit expression of a target gene, in which the dsRNA I has a double-strand structure from, at most, 49 consecutive nucleotide pairs, and in which one strand (as1) or at least a section of one strand (as1) of the double-strand structure is complementary to the sense strand of target gene, and in which the dsRNA I has an overhang formed from 1 to 4 nucleotides on at least one end.

The preceding comments are referred to for the further advantageous embodiment of dsRNA I/II.

The invention is further explained on an example below, with reference to the drawings and practical examples. In the drawings:

Fig. 1a, b schematically depicts a first and second double-strand RNA and

Fig. 2 schematically depicts a target gene,

Fig. 3 depicts relative YFP fluorescence after application of different dsRNA in NIH/3T3 cells (first experiment),

Fig. 4 shows relative YFP fluorescence after application of different dsRNA in NIH/3T3 cells (second experiment),

Fig. 5 shows relative YFP fluorescence after application of different dsRNA in NIH/3T3 cells (third experiment),

Fig. 6 shows relative YFP fluorescence after application of different dsRNA in NIH/3T3 cells (fourth experiment),

Fig. 7 shows relative YFP fluorescence after application of different dsRNA in HeLa-S3 cells (fifth experiment),

Fig. 8 shows fluorescence micrographs of NIH/3T3 cells after transfection with pcDNA-YFP and after cotransfection with pcDNA-YFP and different dsRNAs,

Fig. 9 shows fluorescence micrographs of HeLa-S3 cells after transfection with pcDNA-YFP and after cotransfection with pcDNA-YFP and different dsRNAs,

Fig. 10 shows gel electrophoretic separation of S1 after incubation in mouse serum,

Fig. 11 shows gel electrophoretic separation of S1 after incubation in human serum,

Fig. 12 shows gel electrophoretic separation of S7 after incubation in mouse serum,

Fig. 13 shows gel electrophoretic separation of S7 after incubation in human serum,

Fig. 14 shows gel electrophoretic separation of K3 after incubation in mouse serum,

Fig. 15 shows gel electrophoretic separation of PKC1/2 after incubation in mouse serum,

Fig. 16 shows gel electrophoretic separation of S1A/S4B after incubation in human serum,

Fig. 17 shows gel electrophoretic separation of K2 after incubation in human serum and

Fig. 18 shows GFP-specific immunoperoxidase staining on kidney paraffin sections of transgenic GFP mice,

Fig. 19 shows GFP-specific immunoperoxidase staining on heart paraffin sections of transgenic GFP mice,

Fig. 20 shows GFP-specific immunoperoxidase staining on pancreas paraffin sections transgenic GFP mice,

Fig. 21 shows Western blot analysis of GFP expression in plasma,

Fig. 22 shows Western blot analysis of GFP expression in the kidneys,

Fig. 23 shows Western blot analysis of GFP expression in the heart,

Fig. 24 shows Western blot analysis of EGFR expression in U-87 MG glioblastoma cells,

Fig. 25a shows Northern blot analysis of the MDRI mRNA levels in colon carcinoma cell line LS174T, in which the cells were harvested after 74 hours,

Fig. 25b shows quantification of the band according to Fig. 25a, in which the averages from two values are shown,

Fig. 26a shows Northern blot analysis of the MDRI mRNA levels in colon carcinoma cell line LS174T, in which the cells were harvested after 48 hours,

Fig. 26b shows quantification of the band according to Fig. 26a, in which the average values from the two values are shown,

Fig. 27 shows a comparative depiction of a transmitted light and fluorescence photomicrograph of a transfection with 175 nM dsRNA (sequence R1 in Table 4).

The double-strand ribonucleic acids dsRNA I and dsRNA II, depicted schematically in Figures 1a and 1b, each have a first end E1 and a second end E2. The first and second ribonucleic acids dsRNA I/dsRNA II have single-strand sections formed from about 1 to 4 unpaired nucleotides on their two ends E1 and E2. Two possible variants are shown (variants 1 and 2), in which variant 2 has a smooth end (E2). The smooth ends, however, can also lie on the other end (E1) in another variant.

A target gene situated on DNA is schematically shown in Fig. 2. The target gene is made recognizable by a black bar. It has a first region B1 and a second region B2.

Each strand of the first dsRNA I (as1) and the second dsRNA II (as2) is complementary to the corresponding region B1 or B2 on the target gene.

Expression of the target gene is then inhibited with particular efficiency, if the dsRNA I/dsRNA II has single strand sections on its ends E1, E2. The single-strand sections can be formed both on

strand as1 or as2 and on the counterstrand (ss1 or ss2) or on strand as1, as2 and on the counterstrand.

The regions B1 and B2, as shown in Fig. 2, can be spaced from each other. However, they can also be adjacent to each other or overlap.

I. Inhibition of Expression of the YFP Gene in Fibroblasts:

Double-strand RNAs (dRNAs) were prepared from sequences of the yellow fluorescent protein (YFP), a variant of the GFP (green-fluorescent protein) of the alga *Aequoria victoria* and microinjected into fibroblasts, together with a YFP-coding plasmid. Fluorescence reduction was then evaluated relative to cells without dsRNA.

Experimental Protocol:

By means of an RNA synthesizer (type Expedite 8909, Applied Biosystems, Weiterstadt, Germany) and ordinary chemical methods, the RNA single strands apparent from the sequence protocols SQ148, 149 and SQ159 and the single strands complementary to them were synthesized. Purification then occurred by HPLC. Hybridization of the single strands to a double-strand occurred by heating the stoichiometric mixture of single strands in 10 mM sodium phosphate buffer, pH 6.8, 100 mM NaCl, to 90°C and subsequent cooling over 6 hours to room temperature. The dsRNAs so obtained were microinjected into the test cell.

The murine fibroblast cell line NIH/3T3, ECACC no. 930615624 (European Collection of Animal Cell Cultures) served as test system for these cell culture experiments. The plasmid pcDNA-YFP was used for microinjections, which contained an 800 bp large Bam HI/Eco RI-YFP fragment in the corresponding restriction cleavage sites of the vector pcDNA3. Expression of YFP was investigated under the influence of simultaneously cotransfected sequence-homologous dsRNA. Evaluation occurred under the fluorescence microscope, at the earliest, 3 hours after injection, with reference to green fluorescence.

Preparation of the Cell Cultures:

Cultivation of the cells occurred in DMEM with 4.5 g/L glucose, 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin/streptomycin (100 IU/100 µg/mL, Biochrom) in an incubator under a 5% CO_2 atmosphere at 37°C. The cells underwent passage every 3 days, in order to keep them in the exponential growth phase. A day before performance of transfection, the cells were trypsinized (10 × trypsin/TEDTA, Biochrom) and inoculated with a cell density of 0.73×10^5 cells into coated petri dishes (CORNING® Cell Culture Dish, 35 mM, Corning Inc., Corning, USA). The petri dishes were incubated with 0.2% gelatin (Biochrom) for at least 30 minutes at 37°C, washed once with PBS and immediately used for seeding of the cells. To permit recovery of individual cells, CELLocate coverslips from the Eppendorf company were used (square size 55 µm).

Microinjection:

To perform microinjection, the petri dishes were removed from the incubator for about 10 minutes. About 50 cells were microinjected per dish and batch (FemtoJet; Mikromanipulator 5171, Eppendorf). Glass capillaries (FemtoTip) from the Eppendorf company with a tip inside diameter of 0.5 µm were used for microinjection. The injection time was 0.8 seconds and the pressure 30 hPa. The microinjections were conducted on an Olympus IX50 microscope with a fluorescence device. 14 mM NaCl, 3 mM KCl, 10 mM KH_2PO_4, pH 7.0, was used as injection buffer, which contains 0.01 µg/mL pcDNA-YFP. To check successful microinjection, 0.08% (w/v) Dextran-70000 coupled Texas-Red (Molecular Probes, Leiden, Netherlands) was added to the injection solution. To investigate inhibition of the YFP expression with specific dsRNA, dsRNAs were added to the injection solution: batch 1: 0.1 µM dsRNA (sequence protocol SQ148/149); batch 2: 0.1 µM dsRNA (sequence protocol SQ148/159); batch 3: without RNA. After microinjection, the cells were incubated for at least three more hours in the incubator. The intracellular YFP fluorescence was then evaluated on the microscope: simultaneously red and green-fluorescent cells: microinjection was successful, no inhibition of YFP expression by dsRNA was observed; or control cells were involved, in which no dsRNA were injected; only red fluorescent cells: microinjection was successful, the dsRNA inhibits YFP expression.

Results:

At a dsRNA concentration of 0.1 μM, a significantly increased inhibition or expression of the YFP gene in fibroblasts could be observed during use of dsRNA with the protruding single-strand regions (sequence protocol SQ148/159) on both 3' ends by two nucleotides each, in comparison with dsRNA without protruding single-strand ends (Table 1).

The use of short dsRNA molecules, containing 19-25 base pairs with overhangs of a few, preferably 1 to 3, non-base-paired, single-strand nucleotides, therefore permits comparatively stronger inhibition of gene expression in mammal cells than the use of dsRNAs with the same number of base pairs without the corresponding single-strand overhangs at the same RNA concentration.

Batch	Name	Sequence protocol number	0.1 μM
1	S1A/ S1B	SQ148 SQ149	+
2	S1A/ S4B	SQ148 (protruding end) SQ159	+++
3		without RNA	–

Table 1: The symbols show the relative percentage of non-fluorescent or weakly green fluorescent cells (+++ > 90%; ++ 60-90%; + 30-60%; - < 10%).

II. Inhibition of Gene Expression with Target Gene in Cultivated HELA-S3 Cells and Mouse Fibroblasts by dsRNA:

The efficiency of inhibition of YFP expression after transient transfection of a YFP-coding plasmid based on RNA interference with dsRNAs can be modulated by the configuration of the 3' ends in the length of the base-paired regions.

Practical Example:

To detect the efficiency of dsRNA during specific inhibition of gene expression, transiently transfected NIH/3T3 cells (fibroblasts from NIH Swiss mouse embryo, ECCAC (European Collection of Animal Cell Cultures) no. 93061524) and HELA-S3 (human cervical carcinoma cells, DSMZ (German Collection of Microorganisms and Cell Cultures) no. ACC 161) were used. The plasmid pcDNA-YFP was used for transfection, which contains an 800 bp Bam HI/Eco RI-YFP fragment in the corresponding cleavage sites of the vector pcDNA3. Double-strand RNAs derived from the sequence of the yellow fluorescent protein (YFP) were produced and transiently transfected with the plasmid pcDNA-YFP in the fibroblasts (the employed specific dsRNAs are complementary in their anti-sense strands to the corresponding sections of the gene sequences above YFP and GFP). After 48 hours, the fluorescence reduction was quantified. Cells that were transfected either only with pcDNA-YFP or with pcDNA-YFP and a controlled dsRNA (not derived from the YFP sequence) functioned as controls.

Experimental Protocol:

dsRNA Synthesis:

By means of an RNA synthesizer (type Expedite 8909, Applied Biosystems, Weiterstadt, Germany), and ordinary chemical methods, the RNA individual strands apparent from the sequence protocols and the single strands complementary to them were synthesized. Purification of the crude synthesis products then occurred by means of HPLC. The column NucleoPac PA-100, 9 × 250 mM, from the Dionex company was used; 20 mM tris, 10 mM $NaClO_4$, pH 6.8, 10% acetonitrile as low salt buffer and 20 mM Tris, 400 mM $NaClO_4$, pH 6.8, 10% acetonitrile as high salt buffer. The flow rate was 3 mL/minute. Hybridization of the single strands to a double strand occurred by heating the stoichiometric mixture of the single strands in 10 mM sodium phosphate buffer, pH 6.8, 100 mM NaCl, to 80-90°C and subsequent slow cooling over 6 hours to room temperature.

Seeding of the Cells:

All cell culture work was conducted under sterile conditions in a corresponding work bench (HS18, Hera Safe, Kendro, Heraeus). Cultivation of NIH/3T3 cells and HELA-S3 occurred in an incubator (CO_2 incubatorT20, Hera cell, Kendro, Heraeus) at 37°C, 5% CO_2 and saturated atmospheric humidity in DMEM (Dulbecco's Modified Eagle Medium, Biochrom), for the mouse fibroblasts, and Ham'' F12 for the HELA cells with 10% FCS (fetal calf serum, Biochrom), 2 mM L-glutamine (Biochrom) and penicillin/streptomycin (100 IU/100 µg/mL, Biochrom). In order to keep the cells in the exponential growth phase, the cells underwent passage every 3 days. 24 hours before the forming transfection, the cells were trypsinized (10 × trypsin/EDTA, Biochrom, Germany) and seeded with a cell density of 1.0×10^4 cells/recess into a 96-well plate (Multiwell dishes 96-well flat bottom, Labor Schubert & Weiss GmbH) in 150 µL growth medium.

Performance of Transient Transfection:

Transfection was conducted with Lipofectamine Plus™ reagent (Life Technologies) according to the information of the manufacturer. 0.15 µg pcDNA-YFP plasmid was introduced per well. The total transfection volume was 60 µL. 3-fold samples were used in each case. Plasmid DNA was first complexed, together with dsRNA. For this purpose, the plasmid DNA and the dsRNA were diluted in a serum-free medium and 1 µL PLUS reagent was used per 0.1 µg plasmid DNA (in a volume of 10 µL) and, after mixing for 15 minutes at room temperature, they were incubated. During incubation, 0.5 µL Lipofectamine was diluted in a total of 10 µL serum-free medium per 0.1 µg plasmid DNA, thoroughly mixed, added to the plasmid/dsRNA/PLUS mixture and incubated for another 15 minutes. During incubation, a medium change was conducted. For this purpose, the cells were washed once with 200 µL serum-free medium and then with 40 µL serum-free medium and then incubated further in the incubator, up to addition of DNA/dsRNA/PLUS/Lipofectamine. After addition of 20 µL DNA/dsRNA/PLUS/Lipfectamine per well, the cells were incubated for 2.5 hours in the incubator. After incubation, the cells were then washed once with 200 µL growth medium and incubated for 24 hours until detection of the fluorescence in 200 µL growth medium in the incubator.

Detection of Fluorescence:

24 hours after the last medium change, the fluorescence of the cells was photographed on the fluorescence microscope (IX50-S8F2, fluorescence unit U-ULS100Hg, burner U-RFL-T200, Olympus) with a USH-I02D mercury lamp (USHIO Inc., Tokyo, Japan), equipped with a WIB fluorescence cube and a digital CCD camera (Orca IIIm, Hamamatsu and C4742-95 camera controller). Evaluation of the fluorescence recording occurred with the analysis software 3.1 (Soft Imaging System GmbH, Germany). In order to relate the YFP fluorescence to cell density, a cell nucleus staining was carried out (Hoechst staining). For this purpose, the cells were first fixed for 5 in 100 µL methylcarnoy (75% methanol, 25% glacial acetic acid) and then again for 10 minutes in methylcarnoy. After air drying, the fixed cells were incubated for 30 minutes in the dark with 100 µL per well of Hoechst die (75 ng/mL). After washing twice with PBS (PBS Dulbecco w/o Ca^{2+}, M^{2+}, Biochrom), the Hoechst-stained cells were photographed under the fluorescence microscope (Olympus, WU fluorescence cube for Hoechst).

Figures 3 to 9 show the results on inhibition of YFP expression by dsRNA in the cultivated cells:

The effects of YFP-specific dsRNAs and control dsRNAs on YFP expression in NIH/3T3 mouse fibroblasts after transient transfection are summarized in Figures 3, 4, 5 and 6. The experiments were run as described in the experimental protocol. The concentration of dsRNA refers to the concentration in the medium during the transfection reaction. The designations for the dsRNAs can be gathered from Table 2. The relative fluorescence per image section in area percent is shown. 3 different image sections were evaluated per well. The averages are obtained from the 3-fold batches.

The specific inhibition of YFP gene expression by dsRNAs in HELA-S3 cells is shown in Figures 7 and 9. The inhibiting effect of differently configured dsRNA constructs (Table 2) in different concentrations on expression of YFP in HELA cells is shown in Fig. 7. Fig. 8 shows representative fluorescence microscope recordings of NIH/3T3 mouse fibroblasts transiently transfected with YFP without dsRNA and with dsRNA specifically directed against YFP (× 100 magnification).

8A: YFP controls

8B: S1, 10 nM

8C: S4, 10 nM

8D:S7, 10 nM

8E: S7/S11, 1 nM

8F: S7/S12, 1 nM

Fig. 9 shows representative fluorescence microscope recordings of HELA-3S cells transiently transfected with YFP without dsRNA and with dsRNAs specifically directed against YFP (× 100 magnification).

9A:K2-con trols, 10 nM

9B: S1, 10 nM

9C: S4, 10 nM

9D:S7, 10 nM

9E: S7/11, 1 nM

9F: S7/12, 1 nM

9G:S1A/S 4B, 10 nM

9H: YFP controls

Results:

Fig. 3 shows that YFP expression after transient cotransfection of mouse fibroblasts with the YFP plasmid and dsRNAs specifically directed against the YFP sequence is inhibited with particular efficiency when the 3' ends of the regions containing 22 and 19 base pairs of the dsRNAs have single-strand sections of two nucleotides (nt). Whereas the dsRNA S1 with smooth 3' ends at a concentration of 1 nM (referred to the concentration in the cell culture medium during performance and transfection) exhibits no inhibiting effect on YFP expression, the dsRNAs S7 (19 nucleotide pairs) and S4 (24 nucleotide pairs), each with 2 nt overhangs on both 3' ends, inhibit the YFP expression by 50 or by 70% in comparison with the corresponding control dsRNAs K3 and K2. At a concentration of 10 nM, the dsRNA denoted S1 with smooth ends inhibits YFP expression by about 65%, whereas inhibition of YFP expression by the dsRNA S4 is about 94% (Fig. 4). The inhibiting effects of the dsRNAs denoted S4 and S7 is concentration-dependent (Figures 3 and 4, see also Fig. 7).

Fig. 4 shows that, for efficient suppression of YFP gene expression, the single-strand structure is not necessary on both 3' ends (on the sense and anti-sense strand). To achieve the most effective possible inhibition of YFP expression, only the 2 nt overhang on the 3' end is necessary on the anti-sense strand. Inhibition of YFP expression at a concentration of 1 nM in the two dsRNAs S4 (with 2 nt overhangs on both 3' ends) and S1A/S4B (with the 2 nt overhang on the 3' end of the anti-sense strand) lies at about 70%. On the other hand, if the 2 nt overhang is situated on the 3' end of the sense strand (and the 3' end of the anti-sense strand carries no single-strand region), inhibition of YFP gene expression is only 50%. Similarly, inhibition at higher concentrations is much better, if at least the 3' end of the anti-sense strand carries a 2 nt overhang.

A more distinct inhibition of YFP expression is achieved, if the base-paired region has 21 nucleotide pairs instead of 22 (S1 and S4), 20 (S13 and S13/14) or 19 (S7) (Figures 5, 6 and 7). Inhibition of YFP expression by S1 (22 base pairs with smooth ends) in a concentration of 5 nM is about 40%, whereas inhibition by S7/S12 (21 base pairs with smooth ends), also with 5 nM, lies at about 92%. If the dsRNA with 21 base pairs also has a 2 nt overhang on the anti-sense strand 3' end (S7/S11), inhibition lies at ~ 97% (compared with ~ 73% inhibition with S4 and ~ 70% inhibition with S7).

III. Investigation of Serum Stability of Double-Strand RNA (dsRNA):

The objective is to increase the effectiveness found the cell cultures of inhibition of gene expression of target genes mediated by dsRNA for use in vivo. This is achieved by improved stability of the dsRNAs in serum and by an extended residence time of the molecule in the circulation and the increased effective concentration of the functional molecules related to this, resulting from improved stability.

Practical Example:

The serum stability of dsRNAs that inhibit GFP expression was tested in vivo in murine and human serum.

Experimental Protocol:

Incubation with human or murine serum with the corresponding dsRNA occurred at 37°C. 85 µL serum was incubated with 15 µL 100 µM dsRNA. After specified incubation times (30 minutes, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h), the samples were frozen at -80°C. dsRNA without serum (+ 85 µL ddH_2O) and dsRNA at time 0 were used as control.

For isolation of the dsRNA from the incubation charge, which occurred on ice, 400 µL with 0.1% SDS was added to the charges and these are subjected to phenol extraction: 500 µL phenol:chloroform:isoamyl alcohol (IAA, 25:24:1, Roti®-Phenol, Roth, Karlsruhe) was added per charge and vortexed for 30 seconds at the highest stage (Vortex Genie-2; Scientific Industries). After 10 minutes of incubation on ice, phase separation occurred by centrifuging at 12,000 × g, 4°C for 10 minutes (Sigma 3K30, Rotor 12131-H). The upper aqueous phase (about 200 µL) was taken off and subjected first to DNase I and the proteinase K digestion: addition of 20 µL 10-fold DNase I buffer (100 mM Tris, pH 7.5, 25 mM $MgCl_2$, 1 mM $CaCl_2$) and 10 U DNase I (D7291, Sigma-Aldrich), 30 minutes incubation at 37°C, addition of 6 U DNase I again and incubation for another 20 minutes at 37°C, addition of 5 µL proteinase K (20 mg/mL, 04-1075, Peqlab, Germany) and 30 minutes incubation at 37°C. Phenol extraction was then conducted. For this purpose, 500 µL phenol:chloroform:IAA (25:24:1) was added, vortexed at the highest stage for 30 seconds, 10 minutes for 12000 × g, 4°C, centrifuged, the supernatant taken off and mixed in succession with 40 µL 3M Na-Ac (sodium acetate), pH 5.2, and 1 mL 100% EtOH, mixed thoroughly in the meantime and precipitated for at least 1 hour at -80°C. The precipitate was pelletized by centrifuging at 12000 × g for 30 minutes and 4°C, washed with 70% EtOH and recentrifuged (10 minutes, 12000 × g, 4°C). The air dried pellet was taken up in 30 µL RNA gel application buffer (7 M urea, 1 × TBE (0.09 M tris-borate, 0.002 M EDTA

(ethylenediaminetetraacetate), 0.02% (w/v) bromophenol blue, 0.02% (w/v) xylenecyanol) and stored at -20°C until gel application.

For characterization of the dsRNA, an analytical, denaturing polyacrylamide gel electrophoresis (analytical PAGE) was conducted. The urea gels were prepared right before the run: 7 M urea (21 g) was dissolved during agitation in 25 mL 40% aqueous acrylamide/bisacrylamide stock solution (Rotiphoresis gel, A515.1, Roth) and 5 µL 10 × TBE (100 g Tris, 55 g boric acid, 9.3 g EDTA per L distilled water) and made up to 50 µL with distilled water. Right before pouring, 50 µL TEMED (N,N,N',N'-tetramethylethylenediamine) and 500 µL 10% APS (ammonium peroxidisulfate) were added. After polymerization, the gel was introduced to a vertical electrophoresis apparatus (Merck, Darmstadt) and a prerun was conducted for 30 minutes at a constant 40 mA current intensity. As run buffer, 1 × TBE buffer was used. Before application onto the gel, the RNA samples were heated for 5 minutes at 100°C, cooled on ice and centrifuged for 20 seconds in a table-top centrifuge (Eppendorf, minispin). 15 µL was applied to the gel. The run occurred for about 2 hours at a constant current of 40 mA. After the run, the gel was stained for 30 minutes at RT (room temperature) with Stains all stain solution (20 mL Stains all stock solution dissolved in 200 mL formamide) mixed with 200 mL distilled water and 180 mL formamide) and the background staining eliminated after rinsing in distilled water for 45 minutes. The gels were photographed with the photo documentation system Image Master VDS from Pharmacia.

Figures 10 to 17 show the serum stability of dsRNA after incubation with human and murine serum and subsequent electrophoretic separation in 20% 7 M urea gel.

Fig. 10: Incubation of S2 (0-22-0) in mouse serum

1. at time 0 (without serum)
2. at time 0
3. for 30 minutes
4. for 1 hour
5. for 2hours
6. for 4 hours

7. for 12 hours

8. 2 μL 100 μM S1 without incubation

S1A) Sense strand S1 (10 μL 20 μm S1A)

S1B) Anti-sense strand S1 (10 μL 20 μM S1B)

Fig. 11: Incubation of S1 (0-22-0) in human serum

1. 2 μL 100 μM S1 untreated (without incubation)
2. for 30 minutes
3. for 2 hours
4. for 4 hours
5. for 6 hours
6. for 8 hours
7. for 12 hours
8. for 24 hours

S1A) Sense strand S1 (10 μL 20 μm S1A)

S1B) Anti-sense strand S1 (10 μL 20 μM S1B)

Fig. 12: Incubation at S7 (2-19-20) in mouse serum

1. at time 0 (without serum)
2. for 30 minutes
3. for 4hours
4. for 12 hours

Fig. 13: Incubation of S7 (2-19-2) in human serum

1. Sense strand S7 (10 μL 20 μM S7A)
2. Anti-sense strand S7 (10 μL 20 μM S7B)
3. for 30 minutes
4. for 1 hour
5. for 2 hours
6. for 4 hours
7. for 6 hours

8. for 12 hours
9. for 24 hours
10. at time 0 (without serum)

Fig. 14: Incubation of K3 (2-19-20) in mouse serum

1. Sense strand K3 (10 μL 20 μM K3A)
2. Anti-sense strand K3 (10 L 20 μM K3B)
3. at time 0 (without serum)
4. at time 0 (with serum)
5. for 30 minutes
6. for 1 hour
7. for 2 hours
8. for 4 hours
9. for 12 hours

Fig. 15: Incubation of PKC1/2 (0-22-2) in mouse serum

1. for 30 minutes
2. for 1 hour
3. for 2 hours
4. for 4 hours
5. for 12 hours
6. 2 μL 100 μM PKC1/2 (untreated)

Fig. 16: Incubation of S1A/S4B (0-22-2) in human serum

1. at time 0 (without serum)
2. for 24 hours
3. for 12 hours
4. for 8 hours
5. for 6 hours
6. for 4 hours
7. for 2 hours

8. for 30 minutes
9. Sense strand S1A (10 µL 20 µM S1A)
10. Anti-sense strand S4B (10 µL 20 µM S4B)

Fig. 17: Incubation of K2 (2-22-2) in human serum

1. Sense strand K2 (10 µL 20 µM K2A)
2. Anti-sense strand K2 (10 µL 20 µM K2B)
3. at point 0 (without serum)
4. for 30 minutes
5. for 2 hours
6. for 4 hours
7. for 6 hours
8. for 8 hours
9. for 12 hours
10. for 24 hours

Results:

dsRNAs without single-strand regions on the 3' ends are much more stable in both human and murine serum than dsRNAs with single-strand 2nt overhangs on the 3' ends (Figures 10 to 14 and 17). After 12 and 24 hours incubation at S1 in murine or human serum, bands in the original size are almost fully retained. On the other hand, in dsRNAs with 2nt overhangs on both 3' ends, the stability in human and murine serum diminishes significantly. After only 4 hours of incubation of S7 (Figures 12 and 13) or K3 (Fig. 14), no bands can be detected in the original size.

In order to increase the stability of dsRNA in serum, it is sufficient if the dsRNA has a smooth end. In mouse serum after 4 hours incubation (Fig. 15, track 4), the bands in the original size are scarcely broken down in comparison with S7 (after 4 hours complete degradation; Fig. 12, track 3).

As an optimal compromise with respect to biological efficacy of dsRNA, the use of dsRNA with a smooth end and a single-strand region of 2 nucleotides can be considered, in which the single-strand overhang should be situated on the 3' end of the anti-sense strand.

The sequences used here are apparent from the following Table 2 and the sequence protocols SQ148-151 and 153-167.

Name	Sequenz-protokoll-Nr.	dsRNA-Sequenz	
S1	SQ148 SQ149	(A) 5´- CCACAUGAAGCAGCACGACUUC -3´ (B) 3´- GGUGUACUUCGUCGUGCUGAAG -5´	0-22-0
S7	SQ150 SQ151	(A) 5´- CCACAUGAAGCAGCACGACUU -3´ (B) 3´- CUGGUGUACUUCGUCGUGCUG -5´	2-19-2
K1	SQ153 SQ154	(A) 5´- ACAGGAUGAGGAUCGUUUCGCA -3´ (B) 3´- UGUCCUACUCCUAGCAAAGCGU -5´	0-22-0
K3	SQ155 SQ156	(A) 5´-GAUGAGGAUCGUUUCGCAUGA-3´ (B) 3´-UCCUACUCCUAGCAAAGCGUA-5´	2-19-2
K2	SQ157 SQ158	(A) 5´- ACAGGAUGAGGAUCGUUUCGCAUG -3´ (B) 3´- UCUGUCCUACUCCUAGCAAAGCGU -5´	2-22-2
S1A/ S4B	SQ148 SQ159	(A) 5´- CCACAUGAAGCAGCACGACUUC -3´ (B) 3´- CUGGUGUACUUCGUCGUGCUGAAG -5´	0-22-2

PKC 1/2	SQ160 SQ161	(A) (B)	5´- CUUCUCCGCCUCACACCGCUGCAA -3´ 3´- GAAGAGGCGGAGUGUGGCGACG -5´	2-22-0
S7/S12	SQ150 SQ162	(A) (B)	5´- CCACAUGAAGCAGCACGACUU -3´ 3´- GGUGUACUUCGUCGUGCUGAA -5´	0-21-0
S7/S11	SQ150 SQ163	(A) (B)	5´- CCACAUGAAGCAGCACGACUU -3´ 3´- CUGGUGUACUUCGUCGUGCUGAA -5´	0-21-2
S13	SQ164 SQ165	(A) (B)	5´- CCACAUGAAGCAGCACGACU -3´ 3´- CUGGUGUACUUCGUCGUGCUGA -5´	0-20-2
S13/14	SQ164 SQ166	(A) (B)	5´- CCACAUGAAGCAGCACGACU -3´ 3´- GGUGUACUUCGUCGUGCUGA -5´	0-20-0
S4	SQ167 SQ159	(A) (B)	5´- CCACAUGAAGCAGCACGACUUCUU -3´ 3´- CUGGUGUACUUCGUCGUGCUGAAG -5´	2-22-2
K1A/ K2B	SQ153 SQ158	(A) (B)	5´- ACAGGAUGAGGAUCGUUUCGCA -3´ 3´- UCUGUCCUACUCCUAGCAAAGCGU -5´	0-22-2
K1B/ K2A	SQ154 SQ157	(A) (B)	5´- ACAGGAUGAGGAUCGUUUCGCAUG -3´ 3´- UGUCCUACUCCUAGCAAAGCGU -5´	2-22-0
S1B/ S4A	SQ149 SQ167	(A) (B)	5´- CCACAUGAAGCAGCACGACUUCUU -3´ 3´- GGUGUACUUCGUCGUGCUGAAG -5´	2-22-0

Key to figure: (Headings)

Name

Sequence protocol no.

dsRNA sequence

Table 2

IV. In Vivo Study:

Double-strand RNA (dsRNA) that was derived from the GFP sequence where unspecific dsRNA was injected intravenously into the caudal vein of "GFP laboratory mice" that express the green fluorescent protein (GFP) in all cells that conduct protein biosynthesis. At the end of the experiment, the animals were killed and the GFP expression analyzed in tissue sections and in plasma.

Experimental Protocol:

Synthesis of dsRNA:

By means of an RNA synthesizer (type Expedite 8909, Applied Biosystems, Weiterstadt, Germany), and ordinary chemical methods, the RNA single strands apparent from the sequence protocols and the single strand complementary to them were synthesized. Purification of the crude synthesis products then occurred with HPLC. As columns, NucleoPac PA-100, 9 × 250 mm from the Dionex company were used; 20 mM Tris, 10 mM $NaClO_4$, pH 6.8, 10% acetonitrile was used as low salt buffer and 20 mM Tris, 400 mM $NaClO_4$, pH 6.8, 10% acetonitrile as high salt buffer. The flow rate was 3 mL/minute. Hybridization of the single strands to a double strand occurred by heating of the stoichiometric mixture of the single strands in 10 mM sodium phosphate buffer, pH 6.8, 100 mM NaCl, to 80-90°C and subsequent slow cooling over 6 hours to room temperature.

Experimental Animal Keeping and Performance of the Experiment

The transgenic laboratory mouse strain TgN (GFPU) 5Nagy (Jackson Laboratory, Bar Harbor, Maine, USA) was used, which expresses GFP (with a beta-actin promoter and a CMV intermediate early enhancer) in all previously investigated cells (Hadjantonakis AK et al. 1993, Mech. Dev. 76: 79-90; Hadjantonakis AK et al., 1998 Nature Genetics 19: 220-222). GFP-transgenic mice can be clearly distinguished from the corresponding wild types (WT) by means of fluorescence (with a UV hand lamp). For breeding, the corresponding WT was paired with a heterozygous GFP type.

The experiment was performed according to the German Animal Protection Regulations. The animals were kept under controlled environmental conditions in groups of 3-5 animals in type III Makrolon cages from the Ehret company, Emmendingen, at a constant temperature of 22°C and a light-dark rhythm of 12 h. Softwood granulate 8/15 from the Altromin company, Lage, was used as sawdust litter. The animals received tap water and standard feed Altromin 1324 pelletized (Altromin) ad libitum.

To perform the experiment, the heterozygous GFP animals were kept in groups of 3 animals each in cages , as described above. Injections of the dsRNA solution occurred intravenously (IV) into the caudal vein in 12-hour cycles (between 5:30 and 7:00 a.m. and between 5:30 and 7:00 p.m.) over 5 days. The injection volumes were 60 µL for per 10 g of body weight and the dose was 2.5 mg dsRNA and 50 µg per kg of body weight. Division into groups was as follows:

Group A: PBS (phosphate buffered saline) 60 µL per 10 kg of body weight,

Group B: 2.5 mg per kg of body weight of a nonspecific control dsRNA (K1 control with smooth ends and a double-strand region of 22 nucleotide pairs),

Group C: 2.5 mg per kg of body weight of another unspecific control dsRNA (K3 control with 2nt overhangs on both 3' ends and a double-strand region of 19 nucleotide pairs),

Group D: 2.5 mg per kg of body weight dsRNA (specific against GFP, subsequently referred to as S1, with smooth ends and a double-strand region of 22 nucleotide pairs),

Group E: 2.5 mg dsRNA per kg of body weight (specific against GFP, subsequently referred to as S7, with 2nt overhangs on the 3' ends of both strands and a double-strand region of 19 nucleotide pairs)

Group F: 50 µg S1-dsRNA per kg of body weight (i.e., 1/50 of the dose of group D).

After the last injection of a total of 10 injections, the animals were killed after 14-20 h and the organs and blood taken as described.

Organ Removal:

Immediately after killing the animals by CO_2 inhalation, blood and different organs were taken (thymus, lungs, heart, spleen, stomach, intestines, pancreas, brain, kidneys and liver). The organs were rinsed briefly in cold sterile PBS and divided with a sterile scalpel. One part was fixed for immunohistochemical staining in methylcarnoys (MC, 60% methanol, 30% chloroform, 10% glacial acetic acid) for 24 hours, one part was shock frozen for frozen sections and for protein isolation immediately in liquid nitrogen and stored at -80°C, and another smaller part was frozen for RNA isolation in RNAeasy-Protect (Qiagen) at -80°C. The blood was immediately held for 30 minutes on ice after sampling, mixed, centrifuged for 5 minutes at 2000 rpm (Mini spin, Eppendorf), the supernatant removed and stored at -80°C (here referred to as plasma).

Processing of the Biopsies:

After 24 h of fixation of the tissue in MC, the tissue pieces were dehydrated in an increasing alcohol series at RT (room temperature): every 40 minutes 70% methanol, 80% methanol, 2 × 96% methanol and 3 × 100% isopropanol. The tissue was then heated in 100% isopropanol to 60°C in an incubator, then incubated for 1 hour in an isopropanol/paraffin mixture at 60°C and 3 × for 2 hours in paraffin and then imbedded in paraffin. For immunoperoxidase staining, tissue sections with 3 µm section thickness were prepared with a rotary microtome (Leica), mounted on slides (Superfrost, Vogel) and incubated for 30 minutes at 60°C in an incubator.

Immunoperoxidase Staining versus GFP:

The sections were deparaffinized 3 × 5 minutes in xylene, rehydrated in an increasing alcohol series (3 × 3 min 100% ethanol, 2 × 2 min 95% ethanol) and then incubated for 20 minutes in 3% H_2O_2/methanol to block endogenous peroxidases. All incubation steps were conducted subsequently in a moist chamber. After 3 × 3 minutes of washing with PBS, they were incubated

with the first antibody (goat anti-GFP, sc-5384, Santa Cruz, Biotechnology) 1:500 in 1% BSA/PBS overnight at 4°C. Incubation with the biotinylated secondary antibody (donkey anti-goat; Santa Cruz Biotechnology; 1:2000 dilution) occurred for 30 minutes at RT, whereupon they were incubated for 30 minutes with Avidin D Peroxidase (1:2000 dilution, Vector Laboratories). After each antibody incubation, the sections were washed 3 × 3 min in PBS and the buffer residues removed from the sections with wadding. All antibodies were diluted in 1% bovine serum albumin (BSA)/PBS. Staining with 3,3'-diaminobenzidine (DAB) was conducted with the DAB substrate kit (Vector Laboratories) according to the manufacturer's data. As nuclear counterstain, hematoxylin III according to Gill (Merck) was used. After dehydration in a rising alcohol series at 3 × 5 minutes xylene, the sections were covered with Entellan (Merck). Microscopic evaluation of staining occurred with the IX50 microscope from Olympus, equipped with a CCD camera (Hamamatsu).

Protein Isolation from Tissue Pieces:

800 µL isolation buffer (50 mM HEPES, pH 7.5, 150 mM NaCl; 1 mM EDTA; 2.5 mM EGTA; 10% glycerol; 0.1% Tween; 1 mM DTT, 10 mM β-glycerol phosphate; 1 mM NaF; 0.1 mM Na_3VO_4 with a protease inhibitor tablet "Complete" from Roche) were added to the still frozen tissue pieces and homogenized 2 × 30 seconds with an Ultraturrax (DIAX 900, dispersal die 6 G, Heidolph), and cooled in between on ice. After 30 minutes of incubation on ice, they were mixed and centrifuged for 20 minutes at 1000 × g, 4°C (3K30, Sigma). The supernatant was incubated for another 10 minutes on ice, mixed and centrifuged for 20 minutes to 15000 × g, 4°C. A protein determination according to Bradford, 1976, modified after Zor & Selinger, 1996, was conducted with the supernatant with the Roti-Nanoquant system of Roth according to the data of the manufacturer. BSA (bovine serum albumin) in concentrations from 10 to 100 µg/mL was used for the protein calibration line.

SDS Gel Electrophoresis:

Electrophoretic separation of the proteins occurred in a multigel long electrophoresis chamber from Biometra with a denaturing, discontinuous 15% SDS-PAGE (polyacrylamide gel electrophoresis) according to Lämmli (Nature 277: 680-685, 1970). For this purpose, a

separation gel 1.5 mM thick was initially poured: 7.5 mL acrylamide/bisacrylamide (30%, 0.9%), 3.8 mL 1.5 M tris/HCl, pH 8.4, 150 µL 10% SDS, 3.3 mL doubly distilled water, 250 µL ammonium persulfate (10%), 9 µL TEMED (N,N,N',N'-tetramethylenediamine) and coated up to polymerization with 0.1% SDS. The collected gel was then poured: 0.83 µL acrylamide/bisacrylamide (30%/0.9%), 630 µL 1 M Tris/HCl, pH 6.8, 3.4 mL doubly distilled water, 50 µL 10% SDS, 50 µL 10% ammonium persulfate, 5 µL TEMED.

Before application of the gel, the proteins were mixed with a corresponding amount of 4-fold sample buffer (200 mM tris, pH 6.8, 4% SDS, 100 mM DTT (dithiotreithol), 0.02% bromophenol blue, 20% glycerol), denatured for 5 minutes in a heating unit at 100°C, briefly centrifuged after cooling on ice and applied to the gel. The same plasma or protein amounts were used per track (3 µL plasma and 25 µg total protein each). Electrophoresis occurred water-cooled at RT and a constant 50 V. The protein gel marker from Bio-Rad (kaleidoscope prestained standard) was used as length standard.

Western Blot and Immune Detection:

Transfer of the proteins from SDS-PAGE to a PVDF (polyvinyl difluoride) membrane (Hybond-P, Amersham) occurred in the semi-dry method according to Kyhse-Anderson (J. Biochem. Biophys. Methods 10: 203-210, 1984) at RT and a constant current intensity of 0.8 mA/cm^2 for 1.5 h. A Tris/glycine buffer was used as transfer buffer (39 mM glycine, 465 mM Tris, 0.1% SDS and 20% methanol). To check the electrophoretic transfer, both the gels after blotting and the blot membranes after immune detection were stained with Coomassie (0.1% Coomassie G250, 45% methanol, 10% glacial acetic acid). For saturation of nonspecific bonds, the blot membrane after transfer was incubated in 1% skim milk powder/PBS for 1 h at RT. It was then washed three times for 3 minutes with 0.1% Tween-20/PBS. All subsequent antibody incubations and washing steps occurred in 0.1% Tween-20/PBS. Incubation with the primary antibody (goat anti-GFP, sc 5384, Santa Cruz Biotechnology) occurred in a dilution of 1:1000 for 1 h at RT. It was then washed 3 × 5 min and incubated for 1 hour at RT with a secondary antibody (donkey anti-goat IgG horseradish peroxidase labeled, Santa Cruz Biotechnology) in a dilution of 1:1000. Detection occurred with the ECL system from Amersham according to the data of the manufacturer.

Figures 18 to 20 show inhibition of GFP expression after intravenous injection of dsRNA specifically directed against GFP with immunoperoxidase staining relative to GFP on 3 μm paraffin sections. During the experiment, dsRNA directed against GFP with a double-strand region of 22 nucleotide (nt) pairs without overhangs on the 3' ends (D) and the corresponding unspecific control dsRNA (B), as well as dsRNA directed specifically against GFP with a double-strand region containing 19 nucleotide pairs with 2 nt overhangs on the 3' ends (E) and the corresponding nonspecific control dsRNA (C) were applied in 12-hour cycles over 5 days. (F) received 1/50 of the dose of group D. Animals without dsRNA administration (A) and WT animals were investigated as additional controls. Fig. 18 shows inhibition of GFP expression in kidney sections, Fig. 19 in heart tissue and Fig. 20 in pancreas tissue. Figures 21 to 23 show Western blot analyses of GFP expression in plasma and tissues. Inhibition of GFP expression in plasma is shown in Fig. 21, in the kidneys in Fig. 22 and in the heart in Fig. 23. Total protein isolates from different animals are shown in Fig. 23. The same total protein amounts per track were applied. In the animals, to which unspecific control dsRNA was administered (animals of groups B and C), the GFP expression relative to animals that received no dsRNA is not reduced. Animals that received dsRNA specifically directed against GFP with 2 nt overhangs on the 3' ends of both strands and a double-strand region containing 19 nucleotide pairs exhibited a significantly inhibited GFP expression in the investigated tissues (heart, kidneys, pancreas and blood), compared with the untreated animals (Figures 18 to 23). In the animals of groups D and F, in which dsRNA directed specifically against GFP with smooth ends and double-strand region containing 22 nucleotide pairs was administered, only those animals that received dsRNA in a dose of 50 μg/kg of body weight per day exhibited a specific inhibition of GFP expression, which, however, was much less pronounced than that of the animals in group E.
The summarizing evaluation of GFP inhibition in the tissue sections and in Western blot shows that inhibition of GFP expression in blood and the kidneys is strongest (Figures 18, 21 and 22).

V. Inhibition of Gene Expression of EGF Receptor with dsRNA as a Therapeutic Approach in Forms of Cancer with EGFR Overexpression or EGFR-induced Proliferation:

The epidermal growth factor (EGF) receptor (EGFR) belongs to the receptor tyrosine kinases, transmembrane proteins with an intrinsic tyrosine kinase activity, which participate in the control

of a series of cellular processes, like cell growth, cell differentiation, migratory processes or cell vitality (review in: Van der Geer et al. 1994). The EGFR family consists of 4 members, EGFR (ErbB1), HER2 (ErbB2, HER3 (ErbB3) and HER4 (ErbB4) with a transmembrane domain, a cysteine-rich extracellular domain and an intracellular catalytic domain. The sequence of EGFR, a 170 kDa protein, has been known since 1984 (Ullrich et al., 1984).

EGFR is activated by peptide growth factors, like EGF, TGFα (transforming growth factor), amphiregulin, beta-cellulin, HB-EGF (heparin-binding EGF-like growth factor) and neureguline. Ligand bonding induces formation of homo- or heterodimers with subsequent autophosphorylation of cytoplasmic tyrosine (Ullrich & Schlessinger, 1990; Alroy & Yarden, 1997). The phosphorylated amino acids form the bonding sites for a number of proteins that participate in the proximal steps of signal conduction in a complex network. EGFR participates in a wide variety of tumor diseases and is therefore a suitable target for therapeutic approaches (Huang & Harari, 1999). The mechanisms that lead to an aberrant EGFR activation can be due to overexpression, amplification, constituted activation of mutant receptor forms or autocrine loops (Voldborg et al., 1997). An overexpression of EGFR was described for a number of tumors, like breast cancer (Walker & Dearing, 1999), non-small lung carcinoma (Fontanini et al., 1998), pancreatic carcinomas, colon carcinoma (Salomon et al., 1995) and glioblastomas (Rieske et al., 1998). No efficient and specific therapeutic agents have thus far been available for malignant glioblastomas, in particular.

Practical Example:

To demonstrate the efficacy of dsRNA during specific inhibition of EGFR gene expression, U-87 MG cells (human glioblastoma cells), ECCAC (European Collection of Animal Cell Cultures) no. 89081402 were used, which were transfected with dsRNA directed specifically against the EGF receptor (sequence protocol SQ 51). After about 72 hours' incubation, the cells were harvested, the protein isolated and the EGFR expression investigated in the Western blot method.

Experimental Protocol:

dsRNA Synthesis:

By means of an RNA synthesizer (type Expedite 8909, Applied Biosystems, Weiterstadt, Germany) and an ordinary chemical method, the RNA single strands apparent from the sequence protocols and the single strands complementary to them were synthesized. Purification of the crude synthesis products then occurred with HPLC. The column NucleoPac PA-100, 9 × 250 mm, from the Dionex company was used; 20 mM Tris, 10 mM $NaClO_4$, pH 6.8, 10% acetonitrile was used as low salt buffer and 20 mM Tris, 400 mM $NaClO_4$, pH 6.8, 10% acetonitrile as high salt buffer. The flow rate was 3 mL/minute. Hybridization of the single strands to a double-strand occurred by heating of the stoichiometric mixture of the single strands in 10 mM sodium phosphate buffer, pH 6.8, 100 mM NaCl, to 80-90°C and subsequent slow cooling over 6 hours to room temperature.

Seeding of the Cells:

All cell culture work was conducted under sterile conditions and an appropriate work bench (HS18, Her safe, Kendro, Heraeus). Cultivation of U-87 MG cells occurred in an incubator (CO_2 incubator T20, Hera cell, Kendro, Heraeus) at 37°C, 5% CO_2 and saturated atmospheric humidity in DMEM (Dulbecco's modified eagle medium, Biochrom) with 10% FCS (fetal calf serum, Biochrom), 2 mM L-glutamine (Biochrom), 1 mM sodium pyruvate (Biochrom), 1 × NEAA (nonessential amino acids, Biochrom) and penicillin/streptomycin (100 IU/100 μg/mL, Biochrom). In order to keep the cells in the exponential growth phase, the cells were subjected to passage every 3 days. 24 hours before application of dsRNA by transfection, the cells were trypsinized (10 × trypsin/EDTA, Biochrom, Germany) and seeded with a cell density of 5×10^5 cells/well in a 6-well plate (6-well plates, Labor Schubert & Weiss GmbH) in 1.5 mL growth medium.

Application of dsRNA in Cultivated U-87 MG Cells:

Application of dsRNA occurred by transfection with OLIGOFECTAMINE™ reagent (Life Technologies) according to the information of the manufacturer. The total transfection volume was 1 mL. The dsRNA was first diluted in serum-free medium: for this purpose, 0.5 μL of a 20

μm stock solution of dsRNA specifically directed against EGFR and 9.5 μL of a 20 μm stock solution of unspecific dsRNA (K1A/K2B) with 175 μL serum-free medium were diluted per well (200 nM dsRNA in transfection charge and 10 nM specific EGFR-dsRNA). The OLIGOFECTAMINE™ reagent was also diluted in serum-free mediums: 3 μL with 12 μL medium per well and then incubated for 10 minutes at room temperature. The diluted OLIGOFECTAMINE™ reagent was then added to the dsRNAs diluted in medium, mixed and incubated for another 20 minutes at RT. During incubation, a medium change was performed. The cells were washed for this purpose once with 1 mL serum-free medium and incubated further in the incubator with 800 μL serum-free medium, up to addition of dsRNA/OLIGOFECTAMINE™ reagent. After addition of 200 μL dsRNA/OLIGOFECTAMINE™ reagent per well, the cells were further incubated in the incubator to protein isolation.

Protein Isolation:

About 72 hours after transfection, the cells were harvested and protein isolation carried out. For this purpose, the medium was removed and the cell monolayer washed once with PBS. After addition of 200 μL protein isolation buffer (1 × protease inhibitor "Complete", Roche, 50 mM HEPES, pH 7.5, 1509 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 0.1% Tween-20, 1 mM DTT, 10 mM β-glycerol phosphate, 1 mM NaF, 0.1 mM Na_3VO_4), the cells were separated by means of a cell scraper, incubated for 10 minutes on ice, transferred to an Eppendorf reaction vessel and stored at -80°C for at least 30 minutes. After thawing, the lysate was homogenized on stage 3 for 10 seconds with a disperser (DIAX 900, dispersing die 6G, Heidolph Instruments GmbH & Co KG, Schwabach), incubated on ice for 10 minutes and centrifuged for 15 minutes at 14000 × g, 4°C (3K30, Sigma). A protein determination was conducted with the supernatant according to Bradford with the Roti® Nanoquant system from Roth (Roth GmbH & Co., Karlsruhe) according to the information of the manufacturer. For this purpose, 200 μL protein solution in appropriate dilution was mixed with 800 μL 1 × working solution and the extinction measured in semi-microcells at 450 and 590 nm versus distilled water in a Beckman spectrophotometer (DU 250). The corresponding BSA dilutions were used for the calibration line (beaded BSA, Sigma).

SDS Gel Electrophoresis:

Electrophoretic separation of the proteins occurred in a multi-gel long electrophoresis chamber from Biometra with a denaturing, discontinuous 7.5% SDS-PAGE (polyacrylamide gel electrophoresis) according to Lämmli (Nature 277: 680-685, 197). For this purpose, a separation gel was initially poured with 1.5 mM thickness: 3.7 mL acrylamide/bisacrylamide (30%, 0.9%), 3.8 mL 1 M Tris/HCl, pH 8.4, 150 µL 10% SDS, 7.15 mL doubly distilled water, 150 µL ammonium persulfate (10%), 9 µL TEMED (N,N,N',N'-tetramethylenediamine) and coated with 0.1% SDS to polymerization. The collection gel was then poured: 0.83 mL acrylamide/bisacrylamide (30%/0.9%), 630 µL 1 M Tris/HCl, pH 6.8, 3.4 mL doubly distilled water, 50 µL 10% SDS, 50 µL 10% ammonium persulfate, 5 µL TEMED.

For application to the gel, the protein samples were mixed 1:3 with 4 × sample buffer (200 mM tris, pH 6.8, 4% SDS, 100 mM DTT (dithiotreithol), 0.02% bromophenol blue, 20% glycerol), denatured for 5 minutes at 100°C, briefly centrifuged after cooling on ice and applied to the gel. 35 µg total protein was applied per track. The run occurred water cooled at RT and a constant 50 V. The kaleidoscope protein gel marker (BioRad) was used as length standard.

Western Blot and Immunodetection:

Transfer of the proteins from SDS-PAGE to a PVDF (polyvinyl difluoride) membrane (Hybond-P, Amersham) occurred in the semi-dry method according to Kyhse-Anderson (J. Biochem. Biophys. Methods 10: 203-210, 1984) at RT and a constant current intensity of 0.5 mA/cm^2 for 1.5 h. The following were used as transfer buffer: cathode buffer (30 mM Tris, 40 mM glycine, 10% methanol, 0.01% SDS; pH 9.4), anode buffer I (300 mM Tris, pH 10.4, 10% methanol) and anode buffer II (30 mM Tris, pH 10.4, 10% methanol). Before combining the blot stack with 3 mm Whatman paper (Schleicher & Schüll), the gel was incubated in the cathode buffer and the PVDF membrane (30 seconds beforehand in 100% methanol) in anode buffer II (5 min): 2 layers 3 mm paper (anode buffer I), 1 layer 3 mm paper (anode buffer II), PVDF membrane , gel, 3 layers 3 mm paper (cathode buffer). To check electrophoretic transfer, both the gels after blotting and the blot membranes after immunodetection were stained with Coomassie (0.1% Coomassie G250, 45% methanol, 10% glacial acetic acid).

The blot membrane was incubated after transfer in 1% skim milk powder/ PBS/0.1% Tween-20 for 1 h at RT. It was then washed three times for 3 minutes with 0.1% Tween-20/PBS. All subsequent antibody incubations and washing steps occurred in 0.1% Tween-20/PBS. Incubation with the primary antibody (human EGFR extracellular domain, specific goat IgG, catalog no. AF231, R&D Systems) occurred on a rocking device for 2 h at RT and a concentration of 1.5 µg/mL. It was then washed 3 × for 6 minutes and incubated for 1 hour at RT with the secondary antibody (donkey anti-goat IgG horseradish peroxidase label, Santa Cruz Biotechnology) (diluted 1:10000). After washing (3 × 3 min in PBS/0.1% Tween-20), detection occurred immediately with ECL reaction (enhanced chemiluminescence): 200 µL solution A (250 mM luminol, Roth, dissolved in DMSO), 89 µL solution B (90 mM p-coumaric acid, Sigma, dissolved in DMSO) and 2 mL 30% H_2O_2 solution were pipetted into 18 mL distilled water. Depending on the membrane size, 4-6 mL was pipetted directly onto the membrane, incubated for 1 minute at RT and then an x-ray film (Biomax MS, Kodak) applied immediately.

The sequences used here are shown in the following Table 3 and in the sequence protocols S!153, 157, 158, 168-173.

ES-7	SQ168 SQ169	(A) 5´- AACACCGCAGCAUGUCAAGAU -3´ (B) 3´- UUUUGUGGCGUCGUACAGUUC -5´	2-19-2
ES-8	SQ170 SQ171	(A) 5´- AAGUUAAAAUUCCCGUCGCUAU -3´ (B) 3´- CAAUUUUAAGGGCAGCGAUAGU -5´	2^5-19-2^5
ES2A/ ES5B	SQ172 SQ173	(A) 5´- AGUGUGAUCCAAGCUGUCCCAA -3´ (B) 3´- UUUCACACUAGGUUCGACAGGGUU -5´	0-22-2
K2	SQ157 SQ158	(A) 5´- ACAGGAUGAGGAUCGUUUCGCAUG -3´ (B) 3´- UCUGUCCUACUCCUAGCAAAGCGU -5´	2-22-2

K1A/ K2B	SQ153 SQ158	(A) (B)	5´- ACAGGAUGAGGAUCGUUUCGCA -3´ 3´- UCUGUCCUACUCCUAGCAAAGCGU -5´	0-22-2

Table 3

Inhibition of EGFR Expression in U-87 MG Glioblastoma Cells:

24 hours after seeding of the cells, they were transfected with 10 nM dsRNA as stated (oligofectamine). After 72 hours, the cells were harvested and the protein isolated. Separation of the proteins occurred in 7.5% SDS-PAGE. 35 µg total protein was applied per track. Fig. 4 shows the corresponding Western blot analysis, from which it follows that the EGFR expression after transfection in U-87 MG cells can be significantly inhibited relative to the corresponding controls with the dsRNA specifically directed against the EGFR gene with a 2 nt overhang on the 3' end of the anti-sense strand. This inhibition of expression of an endogenous gene by specific dsRNA therefore confirms the results stated in the practical example II concerning inhibition of expression of an artificial gene introduced to the cell after transient transfection. The inhibition of EGFR expression mediated by ES-7 and ES-8 is much lower. The dsRNAs used in Fig. 24 can be gathered from Table 3.

VI. Inhibition of Expression of the Multidrug Resistance Gene 1 (MDR1):

Experimental Protocol:

In vitro detection for blocking of MDR1 expression was conducted in the colon carcinoma cell line LS174T (ATCC – American Type Culture Collection, Tom et al., 1976). It is known of this cell line that expression of MDR1 can be induced by addition of rifampcin to the culture medium (Geick et al., 2001). Transfections were conducted with different commercial transfection kits (Lipofectamine, Oligofectamine, both Invitrogen; TransMessenger, Qiagen), in which the TransMessenger transfection kit also proved to be best suited for this cell line.

To run the RNA interference experiments, 4 short double-strand ribonucleic acids R1-R4 were used, whose sequences are shown in Table 4. The ribonucleic acids are homologous with sections of the coding sequence of MDR1 (sequence protocol SQ 30). Sequences R1-R3 consists of a 22-mer sense and a 24-mer anti-sense strand, in which the forming double-strand has a two nucleotide overhang on the 3' end of the anti-sense strand (0-22-2). The sequence R4 corresponds to R1, but consists of a 19-mer double strand with 2 nucleotide overhangs on each 3' end (2-19-2).

Name	Sequenz-protokoll-Nr.	Sequenz	Position in Datenbank-# AF016535
Seq	SQ141	5'- CCA UCU CGA AAA GAA GUU AAG A-3'	1320-1342
R1	SQ142	3'-UG GGU AGA GCU UUU CUU CAA UUC U-5'	1335-1318
Seq	SQ143	5'- UAU AGG UUC CAG GCU UGC UGU A-3'	2599-2621
R2	SQ152	3'-CG AUA UCC AAG GUC CGA ACG ACA U-5'	2621-2597
Seq	SQ144	5'- CCA GAG AAG GCC GCA CCU GCA U-3'	3778-3799
R3	SQ145	3'-UC GGU CUC UUC CGG CGU GGA CGU A-5'	3799-3776
Seq	SQ146	5'- CCA UCU CGA AAA GAA GUU AAG-3'	1320-1341
R4	SQ147	3'-UG GGU AGA GCU UUU CUU CAA U -5'	1339-1318

			Position in Datenbank-# AF402779
K1A/	SQ153	5'- ACA GGA UGA GGA UCG UUU CGC A-3'	2829-2808
K2B	SQ158	3'-UC UGU CCU ACU CCU AGC AAA GCG U-5'	2808-2831

Key to figure: (Headings)

Name

Sequence Protocol no.

Sequence

Position in database #AF016535

Table 4

The sequences shown in Table 4 are shown again in the sequence protocol as sequences SQ 141-147, 152, 153, 158. The dsRNAs were transfected in a concentration of 175 nM as double charges into the cells, which were seeded the day before in 12-well plates at 3.8×10^5 cells/well. For this purpose, 93.3 µL EC-R buffer (TransMessenger kit, Qiagen, Hilden) was mixed with 3.2 µL Enhancer-R per transfection charged, thoroughly mixed and incubated for 5 minutes at room temperature. After addition of 6 µL TransMessenger transfection reagent, the transfection charges were vigorously mixed for 10 seconds and incubated for 10 minutes at room temperature. In the meantime, the medium was withdrawn from the cells by suction, washed once with PBS (phosphate buffered saline) and 200 µL fresh medium without FCS per well was added to the cells. After 10 minutes of incubation, 100 µL FCS-free medium was pipetted into the transfection charged, mixed and the mixture pipetted dropwise into the cells (the dsRNA concentration of 175 µm refers to 400 µL of medium total volume). The dsRNA/TransMessenger complexes were incubated for 4 hours at 37°C with the cells in FCS-free medium. A medium change was then conducted, in which the fresh medium contained 10 µm Rifampicin and 10% FCS. As control, and unspecific dsRNA sequence, having no homology with the MDR1 gene sequence, was used (K) and a MOCK transfection carried out, which contained all reagents, except dsRNA.

The cells were harvested after 24, 48 and 72 hours and a total RNA extracted with the RNeasy-Mini-Kit from Qiagen. 10 µg total RNA of each sample was separated on a 1% agarose formaldehyde gel electrophoretically, blotted onto a nylon membrane and specific probes, random marked with 5'-α^{32}-P-dCTP, were hybridized exposed first relative to MDR1 and, after stripping of the blot, relative to GAPDH as internal control and exposed on x-ray film.

The x-ray films were digitized (Image Master, VDS Pharmacia) and quantified with the Image-Quant software. Balancing of the MDR1-specific bands with the corresponding GAPDH bands was then carried out.

Results:

Figures 25 and 26 show Northern blots (Figures 25a, 26a) with quantitative evaluation of the MDR1-specific bands after balancing with the corresponding GADPH values (Figures 25b, 26b). A reduction of MDR1-mRNA by up to 55% could be observed in comparison with MOCK transfection and by up to 45% in comparison with unspecific control transfection. After 48 h, a significant reduction of MDR1-mRNA levels was achieved with the dsRNA constructs designated R1, R2, R3 (Table 4). After 48 hours, no significant reduction relative to the controls was observed with the R4 dsRNA constructs (Figures 26a, 26b). After 72 hours, a much stronger reduction of MDR1-mRNA levels was observed with R1, R2 and R2 relative to the controls, in comparison with the 48-hour values (Figures 25a and 25b).

With R4 at this point a significant reduction of MDR1-mRNA levels could also be achieved. The constructs with a 2 nt overhang on the 3' end of the anti-sense strand and a double-strand region of 22 nucleotide pairs therefore reduced the MDR1-mRNA more efficiently than the constructs of the 2 nt overhangs on the 3' ends of both strands (anti-sense and sense strand) and a double-strand region of 19 nucleotide pairs relatively independently of the sequence region homologous to the MDR1 gene (after 48 hours; Fig. 26b). The results therefore confirm the inhibition of EGFR gene expression described in practical example 4 by specific dsRNAs after transfection in U-87 MG cells.

The transfection efficiency was determined in a separate experiment by means of a Texas-Red-labeled DNA oligonucleotide (TexRed-A $(GATC)_5T$; also 175 nM transfected) (Figures 27a, 27b; 400-fold magnification, 48 hours after transfection). The amount is about 50% based on the red fluorescent cells, in comparison with the total cell count. If one considers the transfection rate of the cells at about 50%, the observed reduction of MDR1-mRNA level lies at about 45-55% (compared with the controls), with the conclusion that in all cells that could be transfected

successfully with specific dsRNA, the MDR1-mRNA was almost fully broken down and specifically.

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Claims

1. Method for inhibition of expression of a target gene in a cell, comprising the following steps:

introduction of at least one double-strand ribonucleic acid (dsRNA I) in an amount sufficient to inhibit expression of the target gene,

in which the dsRNA I has a double-strand structure formed from, at most, 49 consecutive nucleotide pairs, and in which one strand (as1) or at least a section of one strand (as1) of the double-strand structure is complementary to the target gene,

and in which the dsRNA has an overhang formed from 1 to 4 nucleotides at least on one end (E1, E2) of dsRNA I.

2. Method according to Claim 1, in which the dsRNA has the overhang on a 3' end of one strand (as1) and/or on the 3' end of the other strand (ss1).

3. Method according to Claim 1 or 2, in which the dsRNA I is formed smooth on one end (E1, E2).

4. Method according to Claim 3, in which the smooth end (E1, E2) contains the 5' end of one strand (as1).

5. Method according to one of the preceding claims, in which the overhang is formed from 1 to 4 nucleotides, preferably 1 or 2 nucleotides.

6. Method according to one of the preceding claims, in which at least one additional double-strand ribonucleic acid (dsRNA II), formed according to the dsRNA I according to one of the preceding claims, is introduced to the cell, in which one strand (as1) or at least a section of one strand (as1) of dsRNA I is complementary to a first region (B1) of the target gene, and in which

an additional strand (as2) or at least a section of the additional strand (as2) of dsRNA II is complementary to a second region (B2) of the target gene.

7. Method according to one of the preceding claims, in which the dsRNA I and/or dsRNA II have a length of less than 25, preferably 19 to 23, consecutive nucleotide pairs.

8. Method according to one of the preceding claims, in which the first region (B1) and the second region (B2) overlap or abut each other in sections.

9. Method according to one of the preceding claims, in which the first region (B1) and the second region (B2) are spaced from each other.

10. Method according to one of the preceding claims, in which the target gene has one of the sequences SQ001 to SQ140.

11. Method according to one of the preceding claims, in which the target gene is chosen from the following group: oncogene, cytokine gene, id-protein gene, prion gene, genes of angiogenesis-inducing molecules, of adhesion molecules and of cell surface receptors, genes of proteins that participate in metastasizing and/or invasive processes, genes of proteinases, as well as apoptosis and cell cycle-regulating molecules.

12. Method according to one of the preceding claims, in which the target gene is the MDR1 gene.

13. Method according to one of the preceding claims, in which one of the sequences SQ141-173 is used as dsRNA I/II and a combined dsRNA construct of the sequences SQ141-173 from two related anti-sense (as1/2) and sense sequences (ss1/2) is used.

14. Method according to one of the preceding claims, in which expression is inhibited according to the principle of RNA interference.

15. Method according to one of the preceding claims, in which the target gene is expressed in pathogenic organisms, preferably in plasmodia.

16. Method according to one of the preceding claims, in which the target gene is a component of a virus or a viroid.

17. Method according to Claim 16, in which the virus is a human pathogenic virus or viroid.

18. Method according to one of the Claims 16, in which the virus or viroid is an animal or plant pathogenic virus or viroid.

19. Method according to one of the preceding claims, in which unpaired nucleotides are substituted by nucleoside thiophosphate.

20. Method according to one of the preceding claims, in which at least one end (E1, E2) of dsRNA I/II is modified, in order to counteract degradation in the cell or dissociation into single strands.

21. Method according to one of the preceding claims, in which the cohesion of the double-strand structure caused by the complementary nucleotide pairs is increased by at least one chemical link.

22. Method according to one of the preceding claims, in which the chemical link is formed by a covalent or ionic bond, hydrogen bridge bond, hydrophobic interactions, preferably van der Waals or stacking interactions, or by metal-ion coordination.

23. Method according to one of the preceding claims, in which the chemical link is formed in the vicinity of one end (E1, E2).

24. Method according to one of the preceding claims, in which the chemical link is formed by means of one or more compound groups, in which the compound groups are preferably poly-(oxyphosphinicooxy-1,3-propanediol) and/or oligoethylene glycol chains.

25. Method according to one of the preceding claims, in which the chemical link is formed by branched nucleotide analogs instead of nucleotides.

26. Method according to one of the preceding claims, in which the chemical link is formed by purine analogs.

27. Method according to one of the preceding claims, in which the chemical link is formed by azabenzene units.

28. Method according to one of the preceding claims, in which, to produce the chemical link, at least one of the following groups is used: methylene blue; bifunctional groups, preferably bis-(2-chloroethyl)-amine; N-acetyl-N'-(p-glyoxyl-benzoyl)-cystamine; 4-thiouracil, psoralene.

29. Method according to one of the preceding claims, in which the chemical link is formed by thiophosphoryl groups applied in the vicinity of the ends (E1, E2) of the double-strand region.

30. Method according to one of the preceding claims, in which the chemical link is produced by triple helix bonds situated in the vicinity of the ends (E1, E2).

31. Method according to one of the preceding claims, in which the dsRNA I/II is enclosed in micellar structures, advantageously in liposomes.

32. Method according to one of the preceding claims, in which the dsRNA I/II is bonded to at least one viral sheath protein originating from the virus, derived from it or synthetically produced, associated with it or enclosed by it.

33. Method according to one of the preceding claims, in which the sheath protein is derived from polyoma virus.

34. Method according to one of the preceding claims, in which the sheath protein contains the virus protein 1 (VP1) and/or the virus protein 2 (VP2) of polyoma virus.

35. Method according to one of the preceding claims, in which, during formation of a capsid or capsid-like structure from the sheath protein, one side faces the interior of the capsid or capsid-like structure.

36. Method according to one of the preceding claims, in which one strand (as1/as2) of dsRNA I/II is complementary to the primary or processed RNA transcript of the target gene.

37. Method according to one of the preceding claims, in which the cell is a vertebrate cell or a human cell.

38. Method according to one of the preceding claims, in which the dsRNA I/II is administered in an amount of, at most, 5 mg per kilogram of body weight per day to a mammal, preferably a human.

39. Method according to one of the preceding claims, in which the dsRNA I/II is taken up in a buffer solution for application.

40. Method according to one of the preceding claims, in which the dsRNA I/II is administered orally or by means of injection or infusion, intravenously, intratumorally, by inhalation, intraperitoneally.

41. Use of a double-strand ribonucleic acid (dsRNA I) to inhibit expression of a target gene in a cell,

in which the dsRNA I has a double-strand structure formed from, at most, 49 consecutive nucleotide pairs, and in which one strand (as1) or at least a section of one strand (as1) of the double-strand structure is complementary to the target gene,

and in which the dsRNA I has an overhang formed from 1 to 4 nucleotides at least on one end (E1, E2).

42. Use according to Claim 41, in which the dsRNA I has the overhang on the 3' end of one strand (as1) and/or on the 3' end of the other strand (ss1).

43. Use according to Claim 41 or 42, in which the dsRNA I is formed smooth on one end (E1, E2).

44. Use according to Claim 43, in which the smooth end (E1, E2) contains the 5' end of one strand (as1).

45. Use according to one of the Claims 41 to 44, in which the overhang is formed from 1 to 4 nucleotides, preferably one or two nucleotides.

46. Use according to one of the Claims 41 to 45, in which one additional double-strand ribonucleic acid (dsRNA II), formed according to the dsRNA I according to one of the Claims 41 to 45, is introduced to the cell, in which the one strand (as1) or at least a section of the one strand (as1) of dsRNA I is complementary to a first region (B1) of the sense strand of the target gene, and in which the other strand (as2) or at least a section of the other strand (as2) of dsRNA II is complementary to a second region (B2) of the target gene.

47. Use according to one of the Claims 41 to 46, in which the dsRNA I and/or the dsRNA II have a length of less than 25, preferably 19 to 23, consecutive nucleotide pairs.

48. Use according to one of the Claims 41 to 47, in which the first (B1) and the second region (B2) overlap or abut each other in sections.

49. Use according to one of the Claims 41 to 48, in which the first (B1) and the second region (B2) are spaced from each other.

50. Use according to one of the Claims 41 to 49, in which the target gene has the sequences S1001 to SQ140.

51. Use according to one of the Claims 41 to 50, in which the target gene is chosen from the following group: oncogene, cytokine gene, id-protein gene, prion gene, genes of angiogenesis-inducing molecules, of adhesion molecules and of cell-surface receptors, genes of proteins that participate in metastasizing and/or invasive processes, genes of proteinases, as well as apoptosis and cell cycle-regulating molecules.

52. Use according to one of the Claims 41 to 51, in which the target gene is the MDR1 gene.

53. Use according to one of the Claims 41 to 52, in which one of the sequences SQ141-173 is used as dsRNA I/II and a dsRNA construct of sequences SQ141-173 combined from two related anti-sense (as1/2) and sense sequences (ss1/2) is used.

54. Use according to one of the Claims 41 to 53, in which expression is inhibited according to the principle of RNA interference.

55. Use according to one of the Claims 41 to 54, in which the target gene is expressed in pathogenic organisms, preferably in plasmodia.

56. Use according to one of the Claims 41 to 55, in which the target gene is a component of a virus or viroid.

57. Use according to Claim 56, in which the virus is a human pathogenic virus or viroid.

58. Use according to Claim 56, in which the virus or viroid is an animal or plant pathogenic virus or viroid.

59. Use according to one of the Claims 41 to 58, in which unpaired nucleotides are substituted by nucleoside thiophosphates.

60. Use according to one of the Claims 41 to 59, in which at least one end (E1, E2) of dsRNA is modified, in order to counteract degradation in the cell or dissociation into single strands.

61. Use according to one of the Claims 41 to 60, in which cohesion of the double-strand structure caused by the complementary nucleotide pairs is increased by at least one chemical link.

62. Use according to one of the Claims 41 to 61, in which the chemical link is formed by a covalent or ionic bond, a hydrogen bridge bond, a hydrophobic interaction, preferably van der Waals or stacking interactions, or by metal ion coordination.

63. Use according to one of the Claims 41 to 62, in which the chemical link is formed in the vicinity of one end (E1, E2).

64. Use according to one of the Claims 41 to 63, in which the chemical link is formed by means of one or more compound groups, in which the compound groups are preferably poly-(oxyphosphinicooxy-1,3-propanediol) and/or oligoethylene glycol chains.

65. Use according to one of the Claims 41 to 64, in which the chemical link is formed by branched nucleotide analogs used instead of nucleotides.

66. Use according to one of the Claims 41 to 65, in which the chemical link is formed by purine analogs.

67. Use according to one of the Claims 41 to 66, in which the chemical link is formed by azabenzene units.

68. Use according to one of the Claims 41 to 67, in which at least one of the following groups is used to produce the chemical link: methylene blue; bifunctional groups, preferably (bis-(2-chloroethyl)-amine; N-acetyl-N'-(p-glyoxyl-benzoyl)-cystamine; 4-thiouracil; psoralene.

69. Use according to one of the Claims 41 to 68, in which the chemical link is formed by thiophosphoryl groups applied in the vicinity of the ends (E1, E2) of the double-strand region.

70. Use according to one of the Claims 41 to 69, in which the chemical link is produced by triple helix bonds situated in the vicinity of ends (E1, E2).

71. Use according to one of the Claims 41 to 70, in which the dsRNA I/II is enclosed in micellar structures, advantageously in liposomes.

72. Use according to one of the Claims 41 to 71, in which the dsRNA I/II is bonded to at least one viral sheath protein originating from a virus, derived from it or a synthetically produced viral sheath protein, associated with it or enclosed by it.

73. Use according to one of the Claims 41 to 72, in which the sheath protein is derived from polyoma virus.

74. Use according to one of the Claims 41 to 73, in which the sheath protein contains the virus protein 1 (VP1) and/or the virus protein 2 (VP2) of polyoma virus.

75. Use according to one of the Claims 41 to 74, in which, during formation of a capsid or capsid-like structure from the sheath protein, one side faces the interior of the capsid or capsid-like structure.

76. Use according to one of the Claims 41 to 75, in which one strand (as1, as2) of dsRNA I/II is complementary to the primary or processed RNA transcript of the target gene.

77. Use according to one of the Claims 41 to 76, in which the cell is a vertebrate cell or a human cell.

78. Use according to one of the Claims 41 to 77, in which the dsRNA I/II is administered in an amount of, at most, 5 mg per kilogram of body weight per day to a mammal, preferably a human.

79. Use according to one of the Claims 41 to 78, in which the dsRNA I/II is taken up in a buffer solution for application.

80. Use according to one of the Claims 41 to 79, in which the dsRNA I/II is administered orally or by injection or infusion intravenously, intraturmorally, by inhalation, intraperitoneally.

81. Medicament to inhibit expression of a target gene in a cell, containing a double-strand ribonucleic (dsRNA I) in an amount sufficient to inhibit expression of a target gene,

in which the dsRNA I has a double-strand structure formed from, at most, 49 consecutive nucleotide pairs,

and in which one strand (as1) or at least one section of one strand (as1) of the double-strand structure is complementary to the target gene,

and in which the dsRNA I has an overhang formed on at least one end (E1, E2) from 1 to 4 nucleotides.

82. Medicament according to Claim 81, in which the dsRNA I has the overhang on the 3' end of one strand (as1) and/or on the 3' end of the other strand (ss1).

83. Medicament according to Claim 81 or 82, in which the dsRNA I is formed smooth on one end (E1, E2).

84. Medicament according to one of the Claims 83, in which the smooth end (E1, E2) contains the 5' end of one strand (as1).

85. Medicament according to one of the Claims 81 to 84, in which the overhang is formed from 1 to 4 nucleotides, preferably 1 or 2 nucleotides.

86. Use according to one of the Claims 81 to 85, containing at least one additional double-strand ribonucleic acid (dsRNA II), formed according to the dsRNA I according to one of the Claims 81 to 85, in which the one strand (as1) or at least a section of the one strand (as1) of dsRNA I is complementary to a first region (B1) of the target gene, and in which the additional strand (as2) or at least a section of the additional strand (as2) of dsRNA II is complementary to a second region (B2) of the target gene.

87. Use according to one of the Claims 81 to 86, in which the dsRNA I and/or the dsRNA II have a length of less than 25, preferably 19 to 23, consecutive nucleotide pairs.

88. Use according to one of the Claims 81 to 87, in which the first (B1) and the second region (B2) overlap or abut each other in sections.

89. Use according to one of the Claims 81 to 88, in which the target gene has one of the sequences S1001 to SQ140.

90. Use according to one of the Claims 81 to 89, in which the target gene is chosen from the following group: oncogene, cytokine gene, id-protein gene, prion gene, genes of angiogenesis-inducing molecules, of adhesion molecules and of cell-surface receptors, genes of proteins that participate in metastasizing and/or invasive processes, genes of proteinases, as well as apoptosis and cell cycle-regulating molecules.

91. Use according to one of the Claims 81 to 90, in which the target gene is the MDR1 gene.

92. Use according to one of the Claims 81 to 91, in which one of the sequences SQ141-173 is used as dsRNA I/II and a dsRNA construct of sequences SQ141-173 combined from two related anti-sense (as1/2) and sense sequences (ss1/2) is used.

93. Use according to one of the Claims 81 to 92, in which expression is inhibited according to the principle of RNA interference.

94. Use according to one of the Claims 81 to 93, in which the target gene is expressed in pathogenic organisms, preferably in plasmodia.

95. Use according to one of the Claims 81 to 94, in which the target gene is a component of a virus or viroid.

96. Use according to Claim 95, in which the virus is a human pathogenic virus or viroid.

97. Use according to Claim 95, in which the virus or viroid is an animal or plant pathogenic virus or viroid.

98. Use according to one of the Claims 81 to 97, in which unpaired nucleotides are substituted by nucleoside thiophosphates.

99. Use according to one of the Claims 81 to 98, in which at least one end (E1, E2) of dsRNA is modified, in order to counteract degradation in the cell or dissociation into single strands.

100. Use according to one of the Claims 81 to 99, in which cohesion of the double-strand structure caused by the complementary nucleotide pairs is increased by at least one chemical link.

101. Use according to one of the Claims 81 to 100, in which the chemical link is formed by a covalent or ionic bond, a hydrogen bridge bond, a hydrophobic interaction, preferably van der Waals or stacking interactions, or by metal ion coordination.

102. Use according to one of the Claims 81 to 101, in which the chemical link is formed in the vicinity of one end (E1, E2).

103. Use according to one of the Claims 81 to 102, in which the chemical link is formed by means of one or more compound groups, in which the compound groups are preferably poly-(oxyphosphinicooxy-1,3-propanediol) and/or oligoethylene glycol chains.

104. Use according to one of the Claims 81 to 103, in which the chemical link is formed by branched nucleotide analogs used instead of nucleotides.

105. Use according to one of the Claims 81 to 104, in which the chemical link is formed by purine analogs.

106. Use according to one of the Claims 81 to 105, in which the chemical link is formed by azabenzene units.

107. Use according to one of the Claims 81 to 106, in which at least one of the following groups is used to produce the chemical link: methylene blue; bifunctional groups, preferably (bis-(2-chloroethyl)-amine; N-acetyl-N'-(p-glyoxyl-benzoyl)-cystamine; 4-thiouracil; psoralene.

108. Use according to one of the Claims 81 to 107, in which the chemical link is formed by thiophosphoryl groups applied in the vicinity of the ends (E1, E2) of the double-strand region.

109. Use according to one of the Claims 81 to 108, in which the chemical link is produced by triple helix bonds situated in the vicinity of ends (E1, E2).

110. Use according to one of the Claims 81 to 109, in which the dsRNA I/II is enclosed in micellar structures, advantageously in liposomes.

111. Use according to one of the Claims 81 to 110, in which the dsRNA I/II is bonded to at least one viral sheath protein originating from a virus, derived from it or a synthetically produced viral sheath protein, associated with it or enclosed by it.

112. Use according to one of the Claims 81 to 111, in which the sheath protein is derived from polyoma virus.

113. Use according to one of the Claims 81 to 112, in which the sheath protein contains the virus protein 1 (VP1) and/or the virus protein 2 (VP2) of polyoma virus.

114. Use according to one of the Claims 81 to 113, in which, during formation of a capsid or capsid-like structure from the sheath protein, one side faces the interior of the capsid or capsid-like structure.

115. Use according to one of the Claims 81 to 114, in which one strand (as1, as2) of dsRNA I/II is complementary to the primary or processed RNA transcript of the target gene.

116. Use according to one of the Claims 81 to 114, in which the cell is a vertebrate cell or a human cell.

117. Use according to one of the Claims 81 to 116, in which the first (B1) and second region (B2) are spaced from each other.

118. Use according to one of the Claims 81 to 117, in which the dsRNA I/II is contained in an amount of, at most, 5 mg per administration unit.

119. Use according to one of the Claims 81 to 118, in which the dsRNA I/II is taken up in a buffer solution.

120. Use according to one of the Claims 81 to 119, in which the dsRNA I/II is administered orally or by injection or infusion intravenously, intraturmorally, by inhalation, intraperitoneally.

121. Method for inhibition of expression of a target gene in a cell, comprising the following steps:

introduction of at least one double-strand ribonucleic acid (dsRNA I) in an amount sufficient to inhibit expression of the target gene,

in which the dsRNA I has a double-strand structure formed from, at most, 49 consecutive nucleotide pairs, and in which one strand (as1) or at least a section of one strand (as1) of the double-strand structure is complementary to the target gene,

and in which the dsRNA has an overhang formed from 1 to 4 nucleotides on at least one end (E1, E2) of dsRNA I.

122. Method according to Claim 1, in which the dsRNA has the overhang on a 3' end of one strand (as1) and/or on the 3' end of the other strand (ss1).

123. Method according to Claim 1 or 2, in which the dsRNA I is formed smooth on one end (E1, E2).

124. Method according to Claim 3, in which the smooth end (E1, E2) contains the 5' end of one strand (as1).

125. Method according to one of the preceding claims, in which the overhang is formed from 1 to 4 nucleotides, preferably 1 or 2 nucleotides.

126. Method according to one of the preceding claims, in which at least an additional double-strand ribonucleic acid (dsRNA II), formed according to the dsRNA I according to one of the

preceding claims, is introduced to the cell, in which one strand (as1) or at least a section of one strand (as1) of dsRNA I is complementary to a first region (B1) of the target gene, and which an additional strand (as2) or at least a section of the additional strand (as2) of dsRNA II is complementary to a second region (B2) of the target gene.

127. Method according to one of the preceding claims, in which the dsRNA I and/or dsRNA II have a length of less than 25, preferably 19 to 23, consecutive nucleotide pairs.

128. Method according to one of the preceding claims, in which the first (B1) and the second region (B2) overlap or abut each other in sections.

129. Method according to one of the preceding claims, in which the first (B1) and the second region (B2) are spaced from each other.

130. Method according to one of the preceding claims, in which the target gene has one of the sequences SQ001 to SQ140.

131. Method according to one of the preceding claims, in which the target gene is chosen from the following group: oncogene, cytokine gene, id-protein gene, prion gene, genes of angiogenesis-inducing molecules, of adhesion molecules and of cell surface receptors, genes of proteins that participate in metastasizing and/or invasive processes, genes of proteinases, as well as apoptosis and cell cycle-regulating molecules.

132. Method according to one of the preceding claims, in which the target gene is the MDR1 gene.

133. Method according to one of the preceding claims, in which one of the sequences SQ141-173 is used as dsRNA I/II and a dsRNA construct of the sequences SQ141-173 combined from two related anti-sense (as1/2) and sense sequences (ss1/2) is used.

134. Method according to one of the preceding claims, in which expression is inhibited according to the principle of RNA interference.

135. Method according to one of the preceding claims, in which the target gene is expressed in pathogenic organisms, preferably in plasmodia.

136. Method according to one of the preceding claims, in which the target gene is a component of a virus or a viroid.

137. Method according to Claim 16, in which the virus is a human pathogenic virus or viroid.

138. Method according to one of the Claims 16, in which the virus or viroid is an animal or plant pathogenic virus or viroid.

139. Method according to one of the preceding claims, in which unpaired nucleotides are substituted by nucleoside thiophosphate.

140. Method according to one of the preceding claims, in which at least one end (E1, E2) of dsRNA I/II is modified, in order to counteract degradation in the cell or dissociation into single strands.

141. Method according to one of the preceding claims, in which the cohesion of the double-strand structure caused by the complementary nucleotide pairs is increased by at least one chemical link.

142. Method according to one of the preceding claims, in which the chemical link is formed by a covalent or ionic bond, hydrogen bridge bond, hydrophobic interactions, preferably van der Waals or stacking interactions, or by metal-ion coordination.

143. Method according to one of the preceding claims, in which the chemical link is formed in the vicinity of one end (E1, E2).

144. Method according to one of the preceding claims, in which the chemical link is formed by means of one or more compound groups, in which the compound groups are preferably poly-(oxyphosphinicooxy-1,3-propanediol) and/or oligoethylene glycol chains.

145. Method according to one of the preceding claims, in which the chemical link is formed by branched nucleotide analogs used instead of nucleotides.

146. Method according to one of the preceding claims, in which the chemical link is formed by purine analogs.

147. Method according to one of the preceding claims, in which the chemical link is formed by azabenzene units.

148. Method according to one of the preceding claims, in which, to produce the chemical link, at least one of the following groups is used: methylene blue; bifunctional groups, preferably bis-(2-chloroethyl)-amine; N-acetyl-N'-(p-glyoxyl-benzoyl)-cystamine; 4-thiouracil, psoralene.

149. Method according to one of the preceding claims, in which the chemical link is formed by thiophosphoryl groups applied in the vicinity of the ends (E1, E2) of the double-strand region.

150. Method according to one of the preceding claims, in which the chemical link is produced by triple helix bonds situated in the vicinity of the ends (E1, E2).

151. Method according to one of the preceding claims, in which the dsRNA I/II is enclosed in micellar structures, advantageously in liposomes.

152. Method according to one of the preceding claims, in which the dsRNA I/II is bonded to at least one sheath protein originating from the virus, derived from it or synthetically produced, associated with it or enclosed by it.

153. Method according to one of the preceding claims, in which the sheath protein is derived from polyoma virus.

154. Method according to one of the preceding claims, in which the sheath protein contains the virus protein 1 (VP1) and/or the virus protein 2 (VP2) of polyoma virus.

155. Method according to one of the preceding claims, in which, during formation of a capsid or capsid-like structure from the sheath protein, one side faces the interior of the capsid or capsid-like structure.

156. Method according to one of the preceding claims, in which one strand (as1/as2) of dsRNA I/II is complementary to the primary or processed RNA transcript of the target gene.

157. Method according to one of the preceding claims, in which the cell is a vertebrate cell or a human cell.

158. Method according to one of the preceding claims, in which the dsRNA I/II is administered in an amount of, at most, 5 mg per kilogram of body weight per day to a mammal, preferably a human.

159. Method according to one of the preceding claims, in which the dsRNA I/II is taken up in a buffer solution for application.

160. Method according to one of the preceding claims, in which the dsRNA I/II is administered orally or by means of injection or infusion, intravenously, intratumorally, by inhalation, intraperitoneally.

161. Use of one of double-strand ribonucleic acid (dsRNA I) to inhibit expression of a target gene in a cell,

in which the dsRNA I has a double-strand structure formed from, at most, 49 consecutive nucleotide pairs, and in which one strand (as1) or at least a section of one strand (as1) of the double-strand structure is complementary to the target gene,

and in which the dsRNA I has an overhang formed from 1 to 4 nucleotides at least on one end (E1, E2).

162. Use according to Claim 41, in which the dsRNA I has the overhang on the 3' end of one strand (as1) and/or on the 3' end of the other strand (ss1).

163. Use according to Claim 41 or 42, in which the dsRNA I is formed smooth on one end (E1, E2).

164. Use according to Claim 43, in which the smooth end (E1, E2) contains the 5' end of one strand (as1).

165. Use according to one of the Claims 41 to 44, in which the overhang is formed from 1 to 4 nucleotides, preferably one or two nucleotides.

166. Use according to one of the Claims 41 to 45, in which one additional double-strand ribonucleic acid (dsRNA II), formed according to the dsRNA I according to one of the Claims 41 to 45, is introduced to the cell, in which the one strand (as1) or at least a section of the one strand (as1) of dsRNA I is complementary to a first region (B1) of the sense strand of the target gene, and in which the other strand (as2) or at least a section of the other strand (as2) of dsRNA II is complementary to a second region (B2) of the target gene.

167. Use according to one of the Claims 41 to 47, in which the dsRNA I and/or the dsRNA II have a length of less than 25, preferably 19 to 23, consecutive nucleotide pairs.

168. Use according to one of the Claims 41 to 47, in which the first (B1) and the second region (B2) overlap or abut each other in sections.

169. Use according to one of the Claims 41 to 48, in which the first (B1) and the second region (B2) are spaced from each other.

170. Use according to one of the Claims 41 to 49, in which the target gene has the sequences S1001 to SQ140.

171. Use according to one of the Claims 41 to 50, in which the target gene is chosen from the following group: oncogene, cytokine gene, id-protein gene, prion gene, genes of angiogenesis-inducing molecules, of adhesion molecules and of cell-surface receptors, genes of proteins that participate in metastasizing and/or invasive processes, genes of proteinases, as well as apoptosis and cell cycle-regulating molecules.

172. Use according to one of the Claims 41 to 51, in which the target gene is the MDR1 gene.

173. Use according to one of the Claims 41 to 52, in which one of the sequences SQ141-173 is used as dsRNA I/II and a dsRNA construct of sequences SQ141-173 combined from two related anti-sense (as1/2) and sense sequences (ss1/2) is used.

174. Use according to one of the Claims 41 to 53, in which expression is inhibited according to the principle of RNA interference.

175. Use according to one of the Claims 41 to 54, in which the target gene is expressed in pathogenic organisms, preferably in plasmodia.

176. Use according to one of the Claims 41 to 55, in which the target gene is a component of a virus or viroid.

177. Use according to Claim 56, in which the virus is a human pathogenic virus or viroid.

178. Use according to Claim 56, in which the virus or viroid is an animal or plant pathogenic virus or viroid.

179. Use according to one of the Claims 41 to 58, in which unpaired nucleotides are substituted by nucleoside thiophosphates.

180. Use according to one of the Claims 41 to 59, in which at least one end (E1, E2) of dsRNA is modified, in order to counteract degradation in the cell or dissociation into single strands.

181. Use according to one of the Claims 41 to 60, in which cohesion of the double-strand structure caused by the complementary nucleotide pairs is increased by at least one chemical link.

182. Use according to one of the Claims 41 to 61, in which the chemical link is formed by a covalent or ionic bond, a hydrogen bridge bond, a hydrophobic interaction, preferably van-der-Waals or stacking interactions, or by metal ion coordination.

183. Use according to one of the Claims 41 to 62, in which the chemical link is formed in the vicinity of one end (E1, E2).

184. Use according to one of the Claims 41 to 63, in which the chemical link is formed by means of one or more compound groups, in which the compound groups are preferably poly-(oxyphosphinicooxy-1,3-propanediol) and/or oligoethylene glycol chains.

185. Use according to one of the Claims 41 to 64, in which the chemical link is formed by branched nucleotide analogs used instead of nucleotides.

186. Use according to one of the Claims 41 to 65, in which the chemical link is formed by purine analogs.

187. Use according to one of the Claims 41 to 66, in which the chemical link is formed by azabenzene units.

188. Use according to one of the Claims 41 to 67, in which at least one of the following groups is used to produce the chemical link: methylene blue; bifunctional groups, preferably (bis-(2-chloroethyl)-amine; N-acetyl-N'-(p-glyoxyl-benzoyl)-cystamine; 4-thiouracil; psoralene.

189. Use according to one of the Claims 41 to 68, in which the chemical link is formed by thiophosphoryl groups applied in the vicinity of the ends (E1, E2) of the double-strand region.

190. Use according to one of the Claims 41 to 69, in which the chemical link is produced by triple helix bonds situated in the vicinity of ends (E1, E2).

191. Use according to one of the Claims 41 to 70, in which the dsRNA I/II is enclosed in micellar structures, advantageously in liposomes.

192. Use according to one of the Claims 41 to 71, in which the dsRNA I/II is bonded to at least one sheath protein originating from a virus, derived from it or a synthetically produced viral sheath protein, associated with it or enclosed by it.

193. Use according to one of the Claims 41 to 72, in which the sheath protein is derived from polyoma virus.

194. Use according to one of the Claims 41 to 73, in which the sheath protein contains the virus protein 1 (VP1) and/or the virus protein 2 (VP2) of polyoma virus.

195. Use according to one of the Claims 41 to 74, in which, during formation of a capsid or capsid-like structure from the sheath protein, one side faces the interior of the capsid or capsid-like structure.

196. Use according to one of the Claims 41 to 75, in which one strand (as1, as2) of dsRNA I/II is complementary to the primary or processed RNA transcript of the target gene.

197. Use according to one of the Claims 41 to 76, in which the cell is a vertebrate cell or a human cell.

198. Use according to one of the Claims 41 to 77, in which the dsRNA I/II is administered in an amount of, at most, 5 mg per kilogram of body weight per day to a mammal, preferably a human.

199. Use according to one of the Claims 41 to 78, in which the dsRNA I/II is taken up in a buffer solution for application.

200. Use according to one of the Claims 41 to 79, in which the dsRNA I/II is administered orally or by injection or infusion intravenously, intraturmorally, by inhalation, intraperitoneally.

201. Medicament to inhibit expression of a target gene in a cell, containing a double-strand ribonucleic (dsRNA I) in an amount sufficient to inhibit expression of a target gene,

in which the dsRNA I has a double-strand structure formed from, at most, 49 consecutive nucleotide pairs,

and in which one strand (as1) or at least one section of one strand (as1) of the double-strand structure is complementary to the target gene,

and in which the dsRNA I has an overhang formed on at least one end (E1, E2) from 1 to 4 nucleotides.

202. Medicament according to Claim 81, in which the dsRNA I has the overhang on the 3' end of one strand (as1) and/or on the 3' end of the other strand (ss1).

203. Medicament according to Claim 81 or 82, in which the dsRNA I is formed smooth on one end (E1, E2).

204. Medicament according to one of the Claims 83, in which the smooth end (E1, E2) contains the 5' end of one strand (as1).

205. Medicament according to one of the Claims 81 to 84, in which the overhang is formed from 1 to 4 nucleotides, preferably 1 or 2 nucleotides.

206. Use according to one of the Claims 81 to 85, containing at least one additional double-strand ribonucleic acid (dsRNA II), formed according to the dsRNA I according to one of the Claims 81 to 85, in which the one strand (as1) or at least a section of the one strand (as1) of dsRNA I is complementary to a first region (B1) of the target gene, and in which the additional strand (as2) or at least a section of the additional strand (as2) of dsRNA II is complementary to a second region (B2) of the target gene.

207. Use according to one of the Claims 81 to 86, in which the dsRNA I and/or the dsRNA II have a length of less than 25, preferably 19 to 23, consecutive nucleotide pairs.

208. Use according to one of the Claims 81 to 87, in which the first (B1) and the second region (B2) overlap or abut each other in sections.

209. Use according to one of the Claims 81 to 88, in which the target gene has one of the sequences S1001 to SQ140.

210. Use according to one of the Claims 81 to 89, in which the target gene is chosen from the following group: oncogene, cytokine gene, id-protein gene, prion gene, genes of angiogenesis-inducing molecules, of adhesion molecules and of cell-surface receptors, genes of proteins that participate in metastasizing and/or invasive processes, genes of proteinases, as well as apoptosis and cell cycle-regulating molecules.

211. Use according to one of the Claims 81 to 90, in which the target gene is the MDR1 gene.

212. Use according to one of the Claims 81 to 90, in which one of the sequences SQ141-173 is used as dsRNA I/II and a dsRNA construct of sequences SQ141-173 combined from two related anti-sense (as1/2) and sense sequences (ss1/2) is used.

213. Use according to one of the Claims 81 to 92, in which expression is inhibited according to the principle of RNA interference.

214. Use according to one of the Claims 81 to 93, in which the target gene is expressed in pathogenic organisms, preferably in plasmodia.

215. Use according to one of the Claims 81 to 94, in which the target gene is a component of a virus or viroid.

216. Use according to Claim 95, in which the virus is a human pathogenic virus or viroid.

217. Use according to Claim 95, in which the virus or viroid is an animal or plant pathogenic virus or viroid.

218. Use according to one of the Claims 81 to 97, in which unpaired nucleotides are substituted by nucleoside thiophosphates.

219. Use according to one of the Claims 81 to 98, in which at least one end (E1, E2) of dsRNA is modified, in order to counteract degradation in the cell or dissociation into single strands.

220. Medicament according to one of the Claims 81 to 99, in which cohesion of the double-strand structure caused by the complementary nucleotide pairs is increased by at least one chemical link.

221. Medicament according to one of the Claims 81 to 100, in which the chemical link is formed by a covalent or ionic bond, a hydrogen bridge bond, a hydrophobic interaction, preferably van der Waals or stacking interactions, or by metal ion coordination.

222. Medicament according to one of the Claims 81 to 101, in which the chemical link is formed in the vicinity of one end (E1, E2).

223. Medicament according to one of the Claims 81 to 102, in which the chemical link is formed by means of one or more compound groups, in which the compound groups are preferably poly-(oxyphosphinicooxy-1,3-propanediol) and/or oligoethylene glycol chains.

224. Medicament according to one of the Claims 81 to 103, in which the chemical link is formed by branched nucleotide analogs used instead of nucleotides.

225. Medicament according to one of the Claims 81 to 104, in which the chemical link is formed by purine analogs.

226. Medicament according to one of the Claims 81 to 105, in which the chemical link is formed by azabenzene units.

227. Medicament according to one of the Claims 81 to 106, in which at least one of the following groups is used to produce the chemical link: methylene blue; bifunctional groups, preferably (bis-(2-chloroethyl)-amine; N-acetyl-N'-(p-glyoxyl-benzoyl)-cystamine; 4-thiouracil; psoralene.

228. Medicament according to one of the Claims 81 to 107, in which the chemical link is formed by thiophosphoryl groups applied in the vicinity of the ends (E1, E2) of the double-strand region.

229. Medicament according to one of the Claims 81 to 108, in which the chemical link is produced by triple helix bonds situated in the vicinity of ends (E1, E2).

230. Medicament according to one of the Claims 81 to 109, in which the dsRNA I/II is enclosed in micellar structures, advantageously in liposomes.

231. Medicament according to one of the Claims 81 to 110, in which the dsRNA I/II is bonded to at least one viral sheath protein originating from a virus, derived from it or a synthetically produced viral sheath protein, associated with it or enclosed by it.

232. Medicament according to one of the Claims 81 to 111, in which the sheath protein is derived from polyoma virus.

233. Medicament according to one of the Claims 81 to 112, in which the sheath protein contains the virus protein 1 (VP1) and/or the virus protein 2 (VP2) of polyoma virus.

234. Medicament according to one of the Claims 81 to 113, in which, during formation of a capsid or capsid-like structure from the sheath protein, one side faces the interior of the capsid or capsid-like structure.

235. Medicament according to one of the Claims 81 to 114, in which one strand (as1, as2) of dsRNA I/II is complementary to the primary or processed RNA transcript of the target gene.

236. Medicament according to one of the Claims 81 to 114, in which the cell is a vertebrate cell or a human cell.

237. Medicament according to one of the Claims 81 to 116, in which the first (B1) and second region (B2) are spaced from each other.

238. Medicament according to one of the Claims 81 to 117, in which the dsRNA I/II is contained in an amount of, at most, 5 mg per administration unit.

239. Medicament according to one of the Claims 81 to 118, in which the dsRNA I/II is taken up in a buffer solution.

240. Medicament according to one of the Claims 81 to 119, in which the dsRNA I/II is administered orally or by injection or infusion intravenously, intraturmorally, by inhalation, intraperitoneally.

//Key to Figures//

Relative Fluoreszenz = Relative fluorescence

Maus = mouse

Mensch = human

MOCK Transfektion = MOCK transfection

Hellfeld = bright field

Variante = variant

Zielgen = target gene

SEQUENZPROTOCKOLL = SEQUENCE PROTOCOL

<120> Method for Inhibition of Expression of a Target Gene

<213> Artificial sequence

<223> Description of artificial sequence sense strand (R1A) of a dsRNA that is homologous to the MDR1 sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (R1B) of a dsRNA strand, complementary to the MDR1 sequence

<213> Artificial sequence

<223> Description of artificial sequence sense strand (R2A) of a dsRNA that is homologous to the MDR1 sequence

<213> Artificial sequence

<223> Description of artificial sequence sense strand (R3A) of a dsRNA that is homologous to the MDR1 gene

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (R3B) of a dsRNA, complementary to the MDR1 sequence

<213> Artificial sequence

<223> Description of artificial sequence sense strand (R4A) of a dsRNA that is homologous to the MDR1 sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (R4B) of a dsRNA, complementary to the MDR1 sequence

<213> Artificial sequence

<223> Description of artificial sequence sense strand (S1A) of a dsRNA that is homologous to the YFP or GFP sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (S1B) of a dsRNA, complementary to the YFP or GFP sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (S7A) of a dsRNA that is homologous to the YFP or GFP sequence

<213> Artificial sequence

<223> Description of artificial sequence sense strand (S7B) of a dsRNA that is homologous to the YFP or GFP sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (R2B) of a dsRNA, complementary to the MDR1 sequence

<213> Artificial sequence

<223> Description of artificial sequence sense strand (K1A) of a dsRNA that is homologous to the 5' UTR of the neomycin sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (K1B) of a dsRNA, complementary to the 5' UTR of the neomycin sequence

<213> Artificial sequence

<223> Description of artificial sequence sense strand (K3A) of a dsRNA that is homologous to the 5' UTR of the neomycin sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (K3B) of a dsRNA, complementary to the 5' UTR of the neomycin sequence

<213> Artificial sequence

<223> Description of artificial sequence sense strand (K2A) of a dsRNA that is homologous to the 5' UTR of the neomycin sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (K2B) of a dsRNA, complementary to the 5' UTR of the neomycin sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (S4B) of a dsRNA, complementary to the YFP or GFP sequence

<213> Artificial sequence

<223> Description of artificial sequence sense strand (PKC1 A) of a dsRNA that is homologous to the protein kinase C sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (PKC2 B) of a dsRNA, complementary to the YFP or GFP sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (S12B) of a dsRNA, complementary to the YFP or GFP sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (S11B) of a dsRNA, complementary to the YFP or GFP sequence

<213> Artificial sequence

<223> Description of artificial sequence sense strand (S13A) of a dsRNA that is homologous to the YFP or GFP sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (S13B) of a dsRNA, complementary to the YFP or GFP sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (S14B) of a dsRNA, complementary to the YFP or GFP sequence

<213> Artificial sequence

<223> Description of artificial sequence sense strand (S13A) of a dsRNA that is homologous to the YFP or GFP sequence

<213> Artificial sequence

<223> Description of artificial sequence sense strand (ES-7A) of a dsRNA that is homologous to the human EGFR sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (ES-7B) of a dsRNA, complementary to the human EGFR sequence

<213> Artificial sequence

<223> Description of artificial sequence sense strand (ES-8A) of a dsRNA that is homologous to the human EGFR sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (ES-8B) of a dsRNA, complementary to the human EGFR sequence

<213> Artificial sequence

<223> Description of artificial sequence sense strand (ES-2A) of a dsRNA that is homologous to the human EGFR sequence

<213> Artificial sequence

<223> Description of artificial sequence anti-sense strand (ES-5B) of a dsRNA, complementary to the human EGFR sequence